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MECHANISMS OF REGULATION OF TAU IRES MEDIATED TRANSLATION

Niza Nemkul

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MECHANISMS OF REGULATION OF

TAU IRES MEDIATED TRANSLATION

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MECHANISMS OF REGULATION OF

TAU IRES MEDIATED TRANSLATION

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Niza Nemkul, B.S.
Houston, Texas

May,2016

This dissertation is dedicated to my family

Mom, Dad, Nisha di, and Ojas.

MECHANISMS OF REGULATION OF

TAU IRES MEDIATED TRANSLATION

Niza Nemkul, B.S.

Advisory Professor: Dr. Leslie Krushel, Ph.D.

The translation of most eukaryotic mRNAs occurs in a cap-dependent manner. However, a subset of mRNAs are capable of initiating translation in a cap-independent manner by utilizing sequences in their 5' UTR called IRES. It was previously shown that the 5' UTR of the tau mRNA contains an IRES. In this study I show that IRES dependent translation of tau IRES is regulated at multiple levels in order to regulate the expression of the tau protein.

Tau protein is ubiquitously expressed but is concentrated in the brain. In this study, I utilized neural and non-neural cell lines to show that tau IRES is utilized differently (in some cases, up to 50% of total tau translation) depending on the cell type. For many IRES containing mRNAs, IRES activity is enhanced in conditions when cap-dependent translation is shut down, such as during cellular stress and mitosis. In this study, I show that tau IRES activity is upregulated during increased iron, poly (I:C), and extracellular Aβ exposure, which are stress conditions commonly observed in neurodegenerative diseases. Further, I show that tau IRES is differentially regulated by various upstream stimuli through their downstream signaling kinases. However, a comparison of the effect of various signaling pathways on tau and APP IRES suggested that the specific regulation of these IRESes occur downstream of mTOR signaling.

Most IRESes require binding by certain non-canonical factors called IRES trans acting factors (ITAFs) for internal initiation. ITAFs can be positive or negative, thus enhancing or inhibiting IRES function. Examination of sequences in the tau 5' UTR led us to analyze four different RNA binding proteins as putative ITAFs for tau. Out of these, I identified two proteins – polypyrimidine tract binding protein (PTB) and neural PTB (nPTB) as inhibitory ITAFs of tau IRES. Altering the expression of PTB and nPTB *in vitro* and in cells negatively influenced tau IRES activity and protein expression.

Along with sequences in the 5' UTR, the sequences in the 3' UTR of an mRNA may also affect its translation, either through direct interaction between the RNA sequences, or through interaction by RNA binding proteins. In this study, I show that the tau 3' UTR enhances IRES-dependent translation of tau, and this interaction requires the entire tau 3'UTR. Overall, I show that the tau IRES is a unique tool utilized by the mRNA to regulate tau protein expression.

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ABBREVIATIONS

4EBP: eukaryotic initiation factor 4E binding protein

Aβ: amyloid beta

AD: Alzheimer's disease

Apaf-1: apoptotic protease inhibiting factor

APP: Amyloid precursor protein

AurA: Aurora kinase A (AURKA)

CDK5: Cyclin dependent kinase 5

CK1: Casein kinase 1

CMV: cytomegalovirus

DAP5: Death associated protein 5 (eIF4G2)

DTT: Dithiothreitol

eIF: eukaryotic initiation factor

EMCV: encephalomyocarditis virus

FGF-2/bFGF: basic fibroblast growth factor

GSK3 β: Glycogen synthase kinase 3 beta

HIF-1 Hypoxia-inducible factor 1

hnRNP: Heterogeneous nuclear ribonucleoproteins

IGF1R: Insulin-Like Growth Factor 1 Receptor

IRES: Internal ribosome entry site

ITAF: IRES trans-acting factors

JNK: c-Jun N-terminal kinase JNK

LEF1: Lymphoid Enhancer-Binding Factor 1

m7G: 7-methyl guanosine

MAPK: Mitogen activated protein kinase

Mek: Mitogen-activated protein kinase kinase (MAP2K1)

MEKK: Mitogen-activated protein kinase kinase kinase 1

MT: microtubule

mTOR: mammalian target of rapamycin

ND: neurodegenerative diseases

NFT: neurofibrillary tangles

NMD: nonsense-mediated decay

nPTB: neural PTB (PTBP2)

PABP: poly(A)-binding protein

PBS: Phosphate-buffered saline

PDCD4: Programmed cell death protein 4

PI3K: Phosphatidylinositol 3-kinase

PIC: pre-initation complex

PKB: protein kinase B

PKC: protein kinase C

PP2A/PP2B/PP1: protein phosphatase 2A/2B/1

PRKX: Protein Kinase, X-Linked

PTB: polypyrimidine tract binding protein 1 (PTBP1)

PTM: post translational modification

PV: poliovirus

qRTPCR: quantitative reverse transcription polymerase chain reaction

RAPTOR: Regulatory associated protein of mTOR

Rictor: RPTOR independent companion of MTOR

RIP: RNA immunoprecipitation

rpS6: ribosomal protein S6

RRM: RNA recognition motif

RSK: ribosomal protein S6 kinase

siRNA: small interfering RNA

S6K: ribosomal protein S6 kinase

TBST: Tris-Buffered Saline and Tween 20

TMEV: Theiler's murine encephalomyelitis virus

ULK2: Unc-51 Like Autophagy Activating Kinase 2

UNR: upstream of N-ras

USP5: Ubiquitin Specific Peptidase 5

UTR : untranslated region

VEGF: Vascular endothelial growth factor

XIAP: X-linked inhibitor of apoptosis protein

Chapter I

Introduction

1.1 Microtubule associated protein tau (MAPT)

Tau is a microtubule-associated protein that is expressed in all tissues, but is more abundant in the brain. There are four microtubule-binding domains in the C-terminal region of tau protein. Consequently, the major function of tau protein is to aid in microtubule assembly, elongation and stabilization. The N-terminal region of the protein is called the projection domain that allows tau interaction with other proteins including other cytoskeletal elements such as actin filaments, which further allow stabilized microtubules to interact with neurofilaments (Kolarova, Garcia-Sierra, Bartos, Ricny, & Ripova, 2012). Additionally, interaction of tau with microtubules and other functional partners suggest roles of tau in signal transduction of neurons, and cell viability (Kolarova et al., 2012; J. Z. Wang & Liu, 2008). Knockdown of tau in neurons causes defects in neurite extension (Dawson et al., 2001), while tau overexpression affects cell polarity and disrupts axonal transport (Rossi et al., 2014) (Dixit, Ross, Goldman, & Holzbaur, 2008).

These data highlight the importance of regulating tau protein expression for proper neuronal function. Misregulated tau is observed in many neurodegenerative diseases (NDs). In these diseases, called tauopathies, tau is hyperphosphorylated, which causes dissociation of tau from microtubules and leads to microtubule disassembly. In addition, hyperphosphorylated tau polymerizes and aggregates into filamentous structures called neurofibrillary tangles (NFTs). Some of the tauopathies include Alzheimer's disease (AD), Pick's disease, fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), myotonic dystrophy, Parkinson's disease, and progressive supranuclear palsy (Delacourte & Buee, 2000).

The expression of the tau protein can be regulated at the levels of transcription, RNA

processing, translation, post-translation, and degradation. Currently, much of research is focused on studying the regulation of tau by phosphorylation, and alternative splicing (an overview of these regulatory mechanisms is described below). However, very little is known about how tau expression is regulated through protein synthesis. In this thesis I present work that is focused on examining how the expression of tau protein is regulated through translation, specifically through internal translation mediated by the tau 5'UTR.

Transcriptional regulation and alternative splicing of tau protein

Transcriptional regulation of tau occurs mainly through the transcription factors AP2 and SP1 (Andreadis, Wagner, Broderick, & Kosik, 1996; Heicklen-Klein & Ginzburg, 2000) (Gao, Tucker, & Andreadis, 2005). The tau promoter is GC rich and lacks a TATA box (Andreadis, Wagner et al. 1996). There is a major transcriptional start site, which is utilized in all tissues. In addition, there is a minor transcriptional start site which is utilized in neural tissues (Andreadis et al., 1996). The resulting 5' untranslated region (UTR) is 240 nucleotides long and GC rich (72%).

The human tau gene is located on chromosome 17q21, and consists of at least 16 exons that are transcribed to produce three transcripts of 2, 6, or 9 kb, which are differentially expressed in the nervous system. The 2 kb transcript is ubiquitously expressed and targets tau to the nucleus. The 6 kb transcript is expressed in the central nervous system, and 9 kb transcript is expressed in the retina and peripheral nervous system (Andreadis, 2005; J. Z. Wang & Liu, 2008). In the human brain, alternative splicing of the 6kb transcript generates six main tau isoforms, as shown in Figure 1.1. All six isoforms have the same 240 nt 5'UTR. These isoforms differ in their inclusion of exons 2, 3, and 10, and produce tau proteins of different sizes. The exclusion of exons 2 and 3, inclusion of exon 2 only, or inclusion of both

3

exons, generate the 0N, 1N, or 2N tau. The presence or absence of exon 10, which encodes

one of the four microtubule binding domains in the C-terminus, generates either the four

repeat (4R) or three repeat (3R) tau (Kolarova et al., 2012; J. Z. Wang & Liu, 2008) (Figure

1.1). The smallest isoform (3R0N) is expressed only in fetal brain, while the other five

isoforms are expressed after birth depending upon neuronal type and stage of development

(Andreadis, 2005; J. Z. Wang & Liu, 2008). In addition, the ratio of 4R:3R is 1:1 in normal

adults, and imbalance of this ratio is implicated in disease pathogenesis (S. Chen, Townsend,

Goldberg, Davies, & Conejero-Goldberg, 2010; Levy et al., 2005).

	Isoform	Amino acids
R1 R3 R4	3R0N	352
E2 · R1 R3 R4	3R21	381
E2 E3 · R1 R3 R4	3R2N	410
R1 R2 R3 R4	4R0N	383
E2 · R1 R2 R3 R4	4R1N	412
N— E2 E3 · R1 R2 R3 R4 —C	4R2N	441
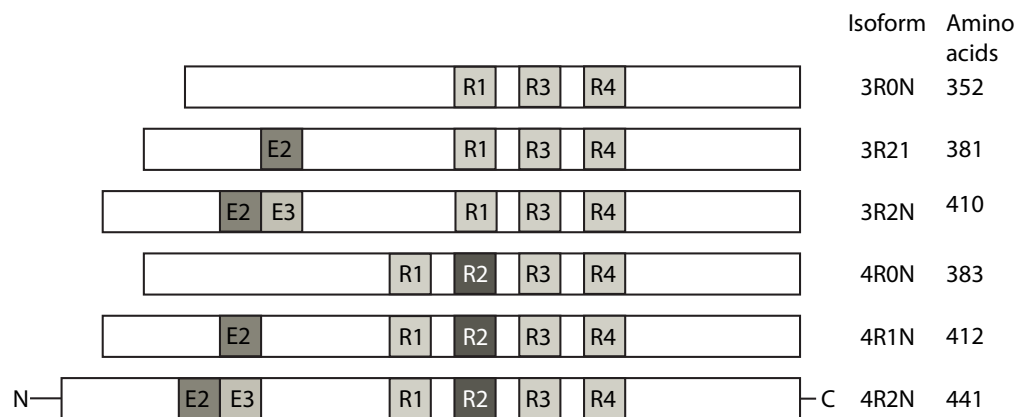
Figure 1.1. **Six isoforms of tau protein in the brain**. Schematic of the alternatively spliced
tau isoforms. Alternative splicing results in the inclusion or exclusion of exon 2, or 3
producing 0N, 1N, or 2N, or of exon 10, which contains a microtubule binding domain (R2)
thus producing 3R, or 4R.

Regulation of tau by post-translational modifications

Tau proteins undergo several post-translational modifications (PTM) including:

phosphorylation, ubiquitylation, glycosylation, glycation, nitration, and truncation. These

modifications are found in both normal as well as diseased conditions, with the exception of

glycation which is only found in tauopathy-affected brain (L. Martin, Latypova, & Terro, 2011).

Tau phosphorylation is the most common and the most studied mechanism for the regulation of tau function. Phosphorylation of tau is thought to play a key role in the developing brain and a lesser role in adult brain, as studies have demonstrated tau phosphorylation is relatively low in normal adult brain. Tauopathies are associated with increased and site specific phosphorylation. The levels of phosphorylation at these different sites, however, are much higher in diseased brain. In some cases, increases in phosphorylation found in brains of AD patients are up to 100-fold higher compared to age-matched control (L. Martin et al., 2011; Yu et al., 2009).

Functionally, phosphorylation with the tau microtubule binding domains decreases interaction with MTs, represses MT assembly, and thus affects axonal transport and neuronal cell structure. Studies suggest that hyperphosphorylation of tau may induce the neuronal cell cycle and is involved in neuron plasticity and maturation (Jovanov-Milosevic et al., 2012). Tau phosphorylation is therefore highly regulated by various kinases and phosphatases. Glycogen synthase kinase 3β (GSK3β), casein kinase 1 (CK1), and cyclin-dependent kinase 5 (CDK5) are thought to be the major kinases that are responsible for aberrant tau hyperphosphorylation observed in NDs. The expression, and activity of these kinases are increased in AD with CK1 mRNA levels up to 30 fold higher compared to control brains. It has been postulated that these kinases work in a sequential pattern, and that phosphorylation by one kinase (such as GSK3β) leads to a kinase cascade allowing other kinases (such as CDK5, or PK1) to phosphorylate tau at other sites (Buee, Bussiere, Buee-Scherrer, Delacourte, & Hof, 2000; L. Martin et al., 2011).

Along with an increase in kinase activity, tau hyperphosphorylation is further augmented by diminished activity of protein phosphatases. Protein phosphatase 2A (PP2A), which is responsible for more than 70% of cellular phosphatase activity, is decreased by 50% in AD brains, while levels of endogenous PP2A inhibitors are increased by 20% (L. Martin et al., 2011). Moreover, FTDP-17 associated mutations are shown to affect binding of tau to PP2A (Goedert et al., 2000). In addition to PP2A, PP2B, PP1, and PP5 are other phosphatases that dephosphorylate tau (F. Liu, Grundke-Iqbal, Iqbal, & Gong, 2005).

Aside from phosphorylation, other PTMs are also observed in NFTs in AD and other tauopathies. Tau protein from NFT brains is polyubiquitinated at lysine 48, however decreased proteasomal activity causes accumulation of these polyubiquitinated tau and formation of tangles. Enhanced levels of N-glycosylation, and nitration of tau is also seen in AD affected brains, while glycation of tau proteins are only found in AD-affected brains. In contrast, there is a reduction in the level of O-GlcNacylation (O-glc-nac) of tau. One reason for this is that O-glc-nac shares the same residues as phosphorylation (L. Martin et al., 2011; Naini & Soussi-Yanicostas, 2015). Therefore it is possible that in normal brains, O-glc-nac may control tau levels by competitively inhibiting phosphorylation.

Tau mutations in tauopathies

A role of tau in neurodegenerative diseases was identified by the observation that mutations in MAPT are associated with FTDP-17 and cause tau pathology (Goedert, Crowther, & Spillantini, 1998; Hutton et al., 1998). At least 37 mutations have been identified in both intronic and exonic regions of the MAPT gene ("AD&FTD Mutation Database," 2015; Wolfe, 2009). Except for two mutations within exon 1, all mutations occur in the C-terminal region encompassing the microtubule-binding domains. These mutations

are either missense mutations that alter tau function, or mutations impacting exon 10 splicing that alter 4R:3R ratio. The increased amount of the 4R tau isoform has been linked to increased filamentous assembly of tau, and 3R isoform is able to inhibit filamentous assembly (Adams, DeTure, McBride, Dickson, & Petrucelli, 2010) (Levy et al., 2005; McMillan et al., 2008). The two mutations in the N-terminal region disrupt binding of tau to the dynactin complex. Other mutations in the MT binding domains reduce tau association with MT, which deregulates MT dynamics, and possibly lead to tau aggregation.

The expressions of the 3R and 4R tau isoforms are highly regulated, and any deviation from the equimolar levels of these isoforms leads to dysregulation of tau function and tau aggregation. Tauopathies including progressive supranuclear palsy, and corticobasal degeneration show increased 4R inclusion. The association of MAPT mutations with various tauopathies emphasizes how tau function is tightly regulated, and suggests that disruption of proper tau protein function is sufficient to induce tau aggregation and neurodegenerative tau phenotype.

Regulation of tau protein expression

In mouse models of tauopathies, reduction of tau expression was shown to alleviate disease symptoms, and even improve neuronal function. For example, transgenic mice expressing mutant P301L through a tetracycline responsive element in the forebrain have severe neurofibrillary pathology including forebrain atrophy and hyper-phosphorylated tau lesions. When the transgene is turned off, these mice show a recession of tau pathology, and enhanced ability to acquire and retain spatial memories (Santacruz et al., 2005; Spires et al., 2006). Reducing tau levels also improves cognitive deficits and memory retention in another AD disease mouse model (hAPP) (Roberson et al., 2007). Similarly, in another Aβ-forming

APP mouse model (APP23), tau reduction prevents memory loss and improves survival (Ittner et al., 2010). It is clear that tau expression is highly controlled and that imbalance in tau expression contributes to disease. Therefore, understanding mechanisms that regulate tau protein expression may help identify novel methodologies for reducing tau levels in neurodegenerative disease states.

1.2 Protein synthesis

The central dogma of molecular biology states that the flow of genetic information occurs from DNA to RNA to proteins, with normal and aberrant regulation of this process playing a role determining the phenotype of an organism. Protein synthesis is a highly regulated process in the gene expression pathway. Numerous studies comparing steady state mRNA of transcribed genes with their expected protein levels have shown that there is not a direct correlation. Instead, studies looking at global mRNA levels and protein abundance show only a causal relationship between the two with only a fraction of protein expression directly related to mRNA levels (Vogel et al., 2010). Thus for most transcribed genes, protein levels appear predominantly controlled at the level of synthesis and degradation (Ghazalpour et al., 2011; Schwanhausser et al., 2011).

Translation of mRNA occurs in three highly regulated steps: initiation, elongation and termination. Since protein synthesis is an energy rich process, it is more efficient for the cell to determine if an mRNA needs to be translated. Therefore initiation is highly regulated and serves as the primary rate-limiting step in translation. The canonical mechanism of translation initiation is called the cap-dependent mechanism. Figure 1.2 provides a schematic of this process.
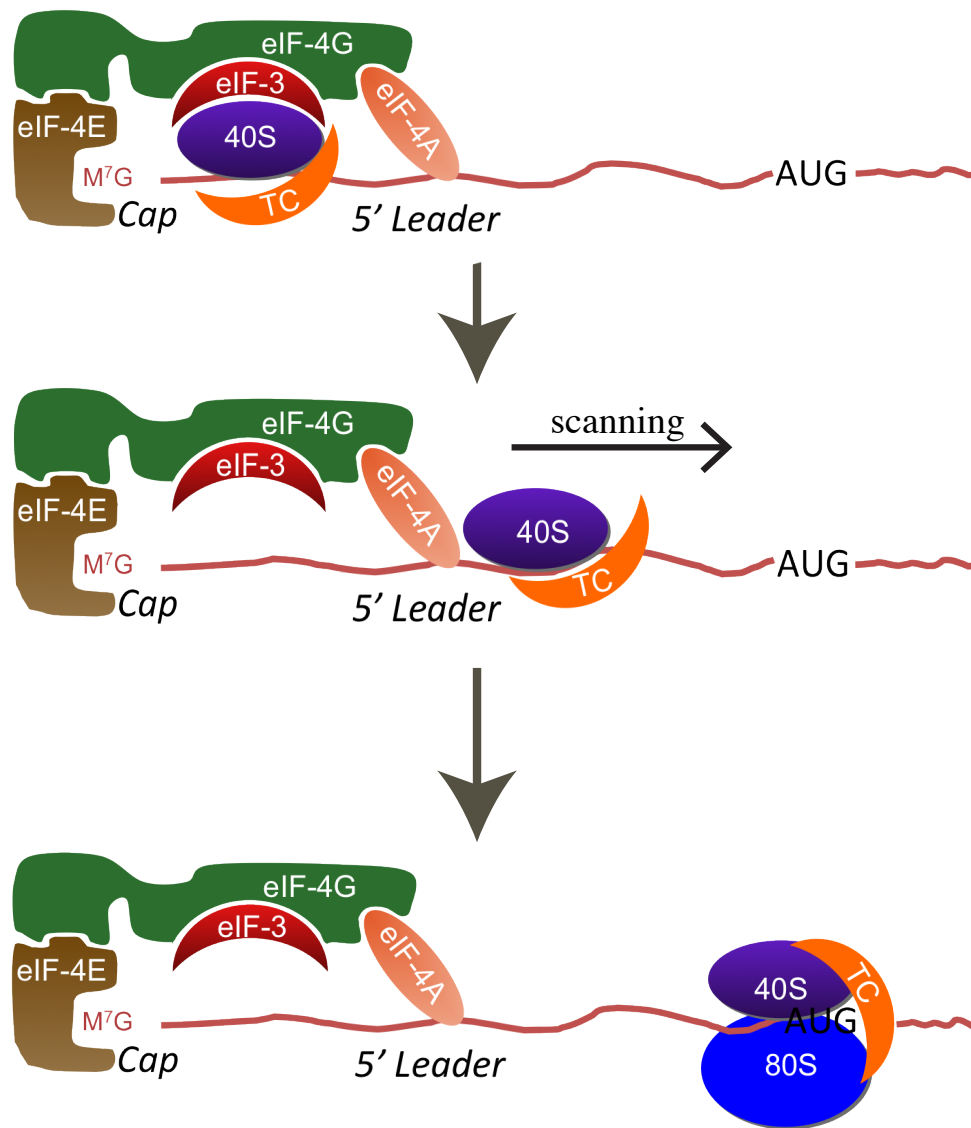
Figure 1.2. **A schematic representation of cap dependent translation initiation.**

The addition of a 7-methyl guanosine (m7G) cap at the 5'end of all eukaryotic mRNAs directs the associations of mRNAs with the eukaryotic initiation factor eIF4E, followed by scaffolding protein eIF4G, and RNA helicase eIF4A forming the cap-binding eIF4F complex. eIF4G contains binding sites for eIF4E, eIF4A, poly(A)-binding protein (PABP), and EIF3. The association of eIF4G and PABP leads to circularization enhancing

stability on the RNA through the creation of a closed loop mRNP structure (not shown). Association of this mRNP complex with eIF3 allows recruitment of the 43S preinitiation complex (PIC). PIC is composed of initiator methionyl-tRNA (Met-tRNA), GTP-bound eIF2, the 40S ribosomal subunit, eIF1, eIF1A, eIF5, and eIF3. The PIC complex then traverses along the 5'UTR to an initiation codon (AUG) whereupon GTP hydrolysis of the complex forms the 48S pre-initiation complex. eIF2, eIF3 and other factors are then released allowing the 60S ribosomal subunit to be recruited via eIF5B with the help of eIF6 to form the 80S initiation complex (IC), which will then begin translation of the coding sequence (Hinnebusch & Lorsch, 2012; Jackson, Hellen, & Pestova, 2010; Sonenberg & Hinnebusch, 2009).

The movement of the PIC down the 5' UTR is called the cap-dependent scanning model of translation initiation. This model is the most widely accepted mechanism of initiation codon selection. According to the scanning hypothesis, the 43S PIC scans along the 5' UTR until it encounters an initiation codon that is in "good context", also called the Kozak sequence (Kozak, 1978). The Kozak consensus sequence is defined by a AUG that is flanked by a purine at the -3 and +4 positions, preferably guanines but adenines will work at the -3 position as well (Rcc**AUGG**)(Cavener, 1987; Kozak, 1987). The scanning mechanism is dependent on the 5' m7G cap for recruiting the ribosome to the mRNA and postulates that complex secondary structure or upstream open reading frames (uORF) would impede translation (Cigan, Pabich, & Donahue, 1988; Kozak, 1979; Kozak & Shatkin, 1978; Mueller & Hinnebusch, 1986). Messages with uORFs are still translated. In this case, the ribosome is thought to bypass start codons that are close to the 5' end in preference for one further downstream by a mechanism called leaky scanning (Kozak, 1987). Another mechanism by

which these uORF-containing mRNAs are translated is through reinitiation. However, reinitiation requires the presence of a stop codon prior to the ORF to allow for termination of the first transcript (Kozak 1987).

The presence of stable hairpin structures that have a free energy of greater than -50 to -60 kcal/mol have been shown to block ribosomal scanning (Kozak, 1986; Pelletier & Sonenberg, 1985). Moreover, the processivity of the ribosome was shown to remain intact on lengthy 5' UTRs as long as it was devoid of additional structures or upstream ORF (uORFs) (Berthelot, Muldoon, Rajkowitsch, Hughes, & McCarthy, 2004). However, it remains largely unclear as to whether the scanning process is subject to specific regulation.

Regulation of cap-dependent initiation by mTOR

The regulation of cap-dependent translation initiation occurs predominantly through actions of the mTOR (mammalian target of rapamycin) signaling pathway. The mTOR pathway primarily regulated through signals transduced through the PI3K pathway and serves to balance regulation mediated through the MAPK and other pathways (Figure 1.3). mTOR is a serine threonine kinase that belongs to the PI3K kinase-related kinase (PIKK) superfamily. The mTOR pathway coordinates cell growth, proliferation, and metabolism in response to various cues derived from upstream signaling pathways, primarily through its regulation of protein synthesis (Inoki, Kim, & Guan, 2012; X. M. Ma & Blenis, 2009). mTOR forms two complexes, mTORC1 and mTORC2, predominantly through the recruitment of scaffolding proteins raptor and rictor, respectively. Both protein complexes share mLST8, Deptor, and Tti1/Tel2 proteins; PRAS40 associates with mTORC1 while mTORC2 contains mSin1 and Protor1/2 proteins. It is the mTORC1 complex that is fundamentally involved in regulation of protein synthesis.
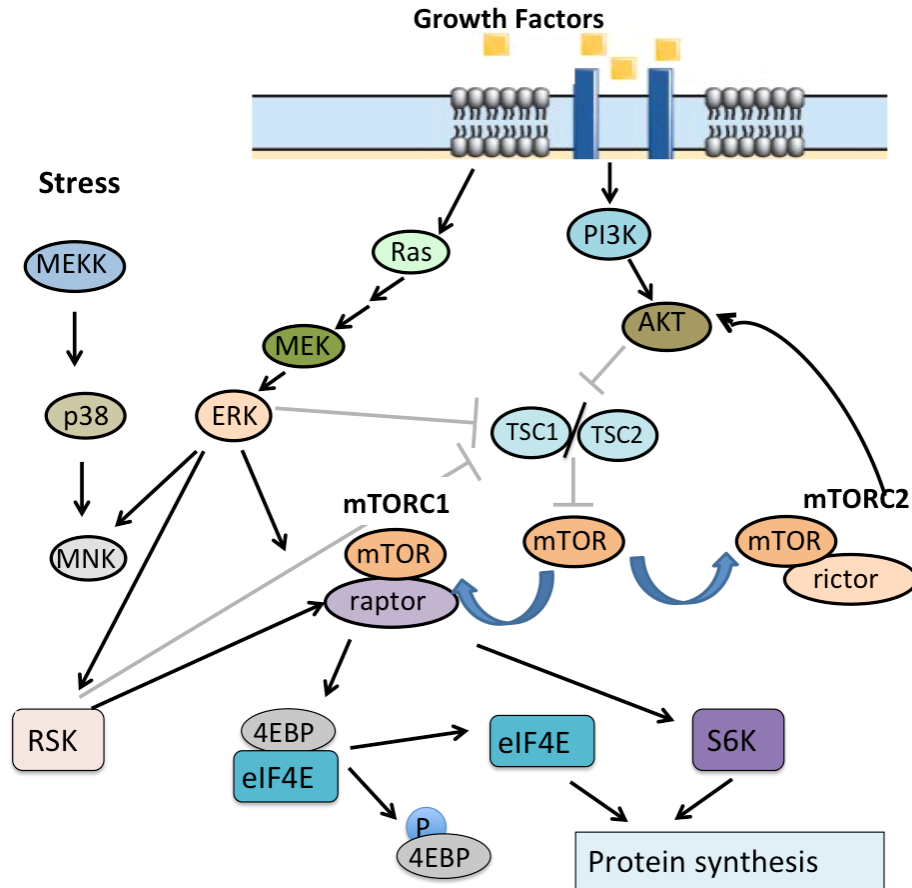
Figure 1.3. **Schematic representation of interacting pathways that regulate protein synthesis**. Growth factors stimulate phosphorylation receptor tyrosine kinases, which activate Ras. Activated Ras then activates MEK through Raf. MEK in turn phosphorylates and activates ERK. ERK inhibits TSC2, and along with RSK, phosphorylates mTOR thus increasing cap-dependent translation. Meanwhile, PI3K is also activated by growth factors, which activates AKT, which promotes mTOR activation through TSC2 inhibition. MTOR exists in two complexes- mTORC1 and mTORC2. mTORC1 regulates protein synthesis either through the 4EBP proteins by regulating eIF4E availability, or through S6K, which phosphorylates ribosomal protein S6. In addition, the MAPK, and Ras-ERK pathways promote cap-dependent translation independently of mTOR, through RSK, which phosphorylates S6 as well.

MTORC1 is activated by growth factors, cytokines or cellular environment, primarily mediated through the MAPK and PI3K signaling pathways (Shaw, 2008). Growth factors and other environmental cues stimulate mTORC1 signaling to control protein synthesis via two main downstream substrates - eIF4E-binding proteins (4E-BPs) and S6 kinases (S6Ks) (Kaizuka et al., 2010; Laplante & Sabatini, 2012; Zoncu, Efeyan, & Sabatini, 2011).

A global regulation of cap-dependent translation is achieved by limiting access of the cap-binding protein eukaryotic initiation factor eIF4E to the m7G cap or to the scaffolding protein eIF4G. EIF4E availability/accessibility is controlled by its interaction with the mTOR target proteins 4E-BP1 and 4E-BP2. In quiescent cells, a hypophosphorylated 4E-BP binds and sequesters eIF-4E, preventing it from binding eIF4G and effectively inhibiting cap-dependent translation. In its activated state mTOR phosphorylates 4E-BP thereby decreasing it affinity with eIF-4E, allowing for cap-dependent translation (Richter & Sonenberg, 2005) [10, 27].

In parallel, mTORC1 acts upon the p70 S6 Kinases (S6K1 and S6K2). Activated mTORC1 phosphorylates S6K1 on its hydrophobic motif, which allows recruitment of PDK1 and subsequent phosphorylation, and activation of S6K1 (Anjum & Blenis, 2008) (X. M. Ma & Blenis, 2009). S6Ks are the primary kinases of ribosomal protein S6 (rpS6), and thereby promote ribosome biogenesis thus controlling overall translational capacity of cells (Meyuhas, 2008). Additionally, multiple S6K substrates are implicated in translation initiation including eIF4B, and PDCD4 via modulation of eIF4A RNA helicase activity (Raught et al., 2004) (Holz, Ballif, Gygi, & Blenis, 2005). Phosphorylated eIF4B acts as a cofactor for eIF4A promoting the RNA helicase activity of eIF4A thus facilitating translation of mRNAs with highly structured 5'UTRs (Holz et al., 2005; G. W. Rogers, Jr., Richter, Lima, & Merrick, 2001).

Unlike mTORC1, the role of mTORC2 in cap-dependent translation initiation is not known. Similar to mTORC1, mTORC2 is activated by environmental cues but mechanism of mTORC2 regulation is not well understood. mTORC2 phosphorylates AKT on Ser473, which is required for full AKT activation. Additionally mTORC2 also phosphorylates and

activates several AGC kinases, including PKB, SGK1, and PKCs (reviewed (Jianling Xie &

Proud, 2014). Thus mTORC2 may indirectly regulate translation through regulation of

mTORC1. It has also been suggested that mTORC2 could indirectly affect protein synthesis

is via eIF2B. mTORC2 activation leads to phosphorylation and inhibition of GSK3β (Case et

al., 2011). Active GSK3β phosphorylates and inhibits eIF2B activity. EIF2B is required for

generating GTP-bound eIF2, which can then bind the initiator Met-tRNAi and promote

translation initiation (X. Wang et al., 2001).

mTOR independent regulation of cap-dependent translation

In addition to regulation by mTOR signaling, other pathways act to regulate cap-

dependent translation initiation by acting on various initiation factors. Along with their

function upstream of mTORC1, the Ras-ERK signaling pathway plays additional regulatory

roles in translation via RSK. Although S6Ks are the primary kinases of rpS6, studies from

S6K1/2 knockout animals have shown that RSK phosphorylates rpS6 at Ser235/236 as well

(Roux et al., 2007), providing an mTOR-independent link between Ras-ERK signaling and

translation initiation. Additionally, RSK phosphorylates other components of the

translational machinery including eIF4B, and PDCD4 (Galan et al., 2014; Shahbazian et al.,

2006), suggesting that AKT and ERK pathways also act directly to control its function in

cap-dependent translation.

The MAPK pathway is also known to regulate 4EBP phosphorylation independent of

mTORC1 through MNK activation by the Ras-ERK as well as p38-MAPK pathways

(Buxade, Parra-Palau, & Proud, 2008). MNKs are then recruited to the eIF4F complex via

binding with eIF4G, thus facilitating the phosphorylation of eIF4E in response to stimulation

by mitogen, or stress (Pyronnet et al., 1999) (Fukunaga & Hunter, 1997; Waskiewicz, Flynn, Proud, & Cooper, 1997).

An additional mechanism of regulation of protein synthesis occurs via eIF2α. As mentioned earlier, the primary role of eIF2 is to mediate the binding of Met-tRNA to the translational machinery. However, in conditions of cellular stress such as viral infection, amino acid deficiency, apoptosis, and hypoxia, eIF2α gets phosphorylated at Ser51 (by PKR, PERK, GCN2, HRI) (Baird & Wek, 2012; Donnelly, Gorman, Gupta, & Samali, 2013). Phosphorylation of eIF2α increases the affinity eIF2 for eIF2B, which prevents ternary complex formation thereby inhibiting protein synthesis (Pavitt, Ramaiah, Kimball, & Hinnebusch, 1998). Moreover, eIF2 phosphorylation also down regulates rRNA transcription, which adds another layer of regulation on protein synthesis (DuRose, Scheuner, Kaufman, Rothblum, & Niwa, 2009).

While these mechanisms lead to a global regulation of cap-dependent protein synthesis, an additional mechanism exists (called IRES dependent translation), which allows specific regulation of translation of a subset of mRNAs.

IRES-dependent translation initiation

Cap-independent translation initiation occurs in a subset of mRNAs through the use of sequences within the 5'UTR termed internal ribosome entry sites (IRES). IRES-dependent translation was first identified for viral RNAs; viral proteins are efficiently translated when host cell cap-dependent translation is inhibited following viral infection (S. K. Jang, Davies, Kaufman, & Wimmer, 1989; S. K. Jang et al., 1988; Pelletier & Sonenberg, 1988). Viruses utilizing IRES-mediated translation employ multiple mechanisms to shut down cap-dependent protein synthesis of the host cell. For example, members of the picornaviridae

family use virus encoded proteases to cleave eIF4G, thereby shutting down cap-dependent translation while still utilizing the carboxy terminal of eIF4G as a scaffold to bring essential factors to the IRES for translation of viral proteins (Lamphear, Kirchweger, Skern, & Rhoads, 1995). Other viruses employ additional mechanisms to shut down host protein synthesis. For example, the encephalomyocarditis virus (EMCV) and poliovirus are able to activate 4EBP function by dephosphorylating 4EBP and reducing cap-dependent translation (Beretta, Gingras, Svitkin, Hall, & Sonenberg, 1996). Finally, coxsackie virus and poliovirus derived proteases are able to cleave PABP, and eIF4G, thus reducing cap-dependent initiation (Kerekatte et al., 1999; Kuyumcu-Martinez, Van Eden, Younan, & Lloyd, 2004).

As an alternative to protein recognition of m7G cap, viral RNAs contain a well characterized IRES structure that serves a point of translation initiation complex formation, in some cases with the requirement of additional factors. Viral IRESes are commonly found in long 5'UTRs (600-1200 nucleotides) and are GC-rich with extensive secondary structures (Balvay, Soto Rifo, Ricci, Decimo, & Ohlmann, 2009; Filbin & Kieft, 2009). Viral IRESes can be classified based on their requirement of canonical factors (Filbin & Kieft, 2009), as shown in Figure 1.4.

Group 1 includes the cricket paralysis virus (CrPV) intergenic IRES and other members of the dicistroviridae intergenic IRESes. This class is the most structurally compact IRESes and are able to facilitate ribosomal recruitment with no initiation factors. Hepatitis C virus, swine fever virus IRES, and members of the flaviviridae family provide examples of Group 2 IRESes. These IRESes require a minimal set of canonical initiation factors such as eIF3, eIF2, and the 40S ribosome (Pestova, Shatsky, Fletcher, Jackson, & Hellen, 1998).
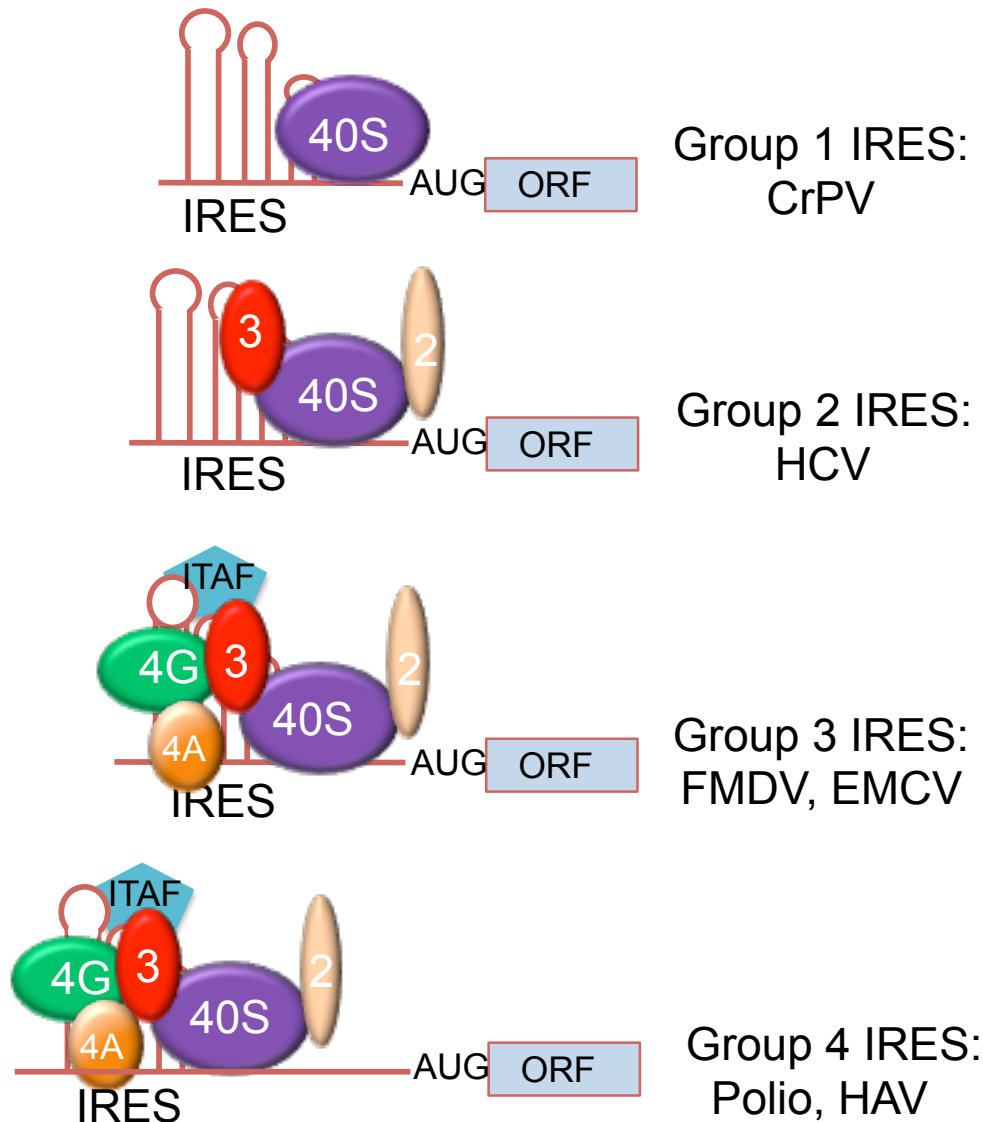
Figure 1.4. **Classifications of viral IRESes.** Viral IRESs can be separated into four different classes based on canonical factor requirements. Group 1: This class of viral IRESes is able to facilitate ribosomal recruitment with no initiation factors at all. Group 2: The second class of viral IRESes requires a minimal set of canonical initiation factors such as eIF3, eIF2, and the 40S ribosomal subunit. Group 3: These IRESes require eIF4G, eIF4A, eIF4B, eIF3, eIF2, and some non-canonical factors (ITAF). Group 4: The last group of IRESes requires some canonical eIFs, and non-canonical factors. Compared to the other groups this one is able to initiate at a start codon a bit downstream of the IRES. (Filbin & Kieft, 2009, modified with permission from Elsevier).

Group 3 viral IRESes include the EMCV and the foot-and-mouth disease virus

(FMDV) as examples. These IRESes require eIF4A and eIF4G along with polypyrimidine

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tract binding protein (PTB) acting as an ITAF to recruit the 40S ribosomal subunit
(Borovjagin, Pestova, & Shatsky, 1994; Lomakin, Hellen, & Pestova, 2000). Group 4
IRESes, which includes polio, hepatitis A, and picornaviridae family viruses, are defined by
a larger 5' UTR and the requirement for eIF4G, eIF4A, eIF4B, eIF3, eIF2, as well as
additional IRES trans-acting factors (ITAFs) (Borman, Michel, & Kean, 2001; Filbin &
Kieft, 2009; Pestova & Hellen, 2000; Pestova, Maslova, Potapov, & Agol, 1989). For this
class scanning and initiating translation occurs a start codon immediately downstream of the
IRES (Kieft, Zhou, Jubin, & Doudna, 2001; Locker, Easton, & Lukavsky, 2007).

Cellular IRES

The first example of cellular IRES was identified in the mRNA encoding the
immunoglobulin heavy-chain binding protein (BiP-1) when it was discovered that the mRNA
continues to be translated even after poliovirus infection when cap-dependent translation was
inhibited (Macejak & Sarnow, 1991). Numerous studies since then have identified additional
cellular mRNAs that contain IRES elements that function to mediate cap-independent
translation. In fact, an estimated 10% of cellular mRNA may be capable of IRES-dependent
translation (Spriggs, Stoneley, Bushell, & Willis, 2008). A list of some known cellular
IRESes is presented in Table 1.1. It was initially predicted that eukaryotic IRESes would
exhibit characteristics that were similar to viral IRESes, such as the length, sequence content,
structure, etc. However, many of defining characteristics of viral IRESes are not observed
for eukaryotic IRESes, and thus bioinformatics prediction of the presence of a eukaryotic
IRES is currently not possible.

Table 1.1. **Cellular mRNAs with Internal Ribosomal Entry Sites classified based on their cellular function.**

Apoptosis	Mitosis	Oncogenes/ Tumor Suppressors	Growth Factors
XIAP	c-myc	c-myc	TrkB
Bcl-xL	ODC	P53	IGF-2
Apaf-1	p58^{PITSLRE}	APC	FGF2
Bip	hnRNP A/B	Pim-1	VEGF
C-myc	unr	p58^{PITSLRE}	PDGF2
c-IAP	p27^{KIP}	C-jun	IGFII
Bcl-2	Aβ	AurKA	
DAP5	NAPIL1	p27^{KIP}	
HIAP2	cyclinD		
Bag-1			

Transcription factors	Signaling	Development	Neuronal
c-jun	PKCδ	MYT2	ARC
c-myc	p58^{PITSLRE}	RUNX1	MAP2
n-myc	p27^{KIP}	Antennapedia	RC3
p53	Notch2	Ultrabithorax	Dendrin
RUNX1	Pim-1	NRF	FMR1
MYT2	Connexin-32		APP
GTX			tau
Mnt			
SMAD-5			
Pim-1			

Our current understanding of eukaryotic IRESes is that they exist in the 5'UTR of mRNAs and can recruit the ribosome without the use of the m7G-cap or the cap-binding protein eIF4E. A schematic of eukaryotic IRES dependent translation is shown in Figure 1.5. One common characteristic among eukaryotic and some viral IRESes is their recognition in response to cellular stress. Cellular stress can inhibit cap-dependent translation in various ways including activating kinases that phosphorylate eIF2α thereby disrupting ternary complex formation. Similarly, other stress conditions can inhibit mTOR activity, activating 4EBPs to sequester eIF4E thereby inhibiting cap-dependent translation. Some examples of cellular IRESes that are regulated in response to stress stimuli include the VEGF mRNA, which was shown to maintain translation during hypoxic conditions (Young et al., 2008).

IRESes in Apaf-1 and XIAP mRNA are upregulated in response to UV irradiation (Holcik &

Sonenberg, 2005; Ungureanu et al., 2006), and examples of regulation in response to mitosis,

nutrient deprivation, and inflammation are also reported (Pyronnet, Pradayrol, & Sonenberg,

2000; Rubsamen et al., 2012; Woeller, Fox, Perry, & Stover, 2007).
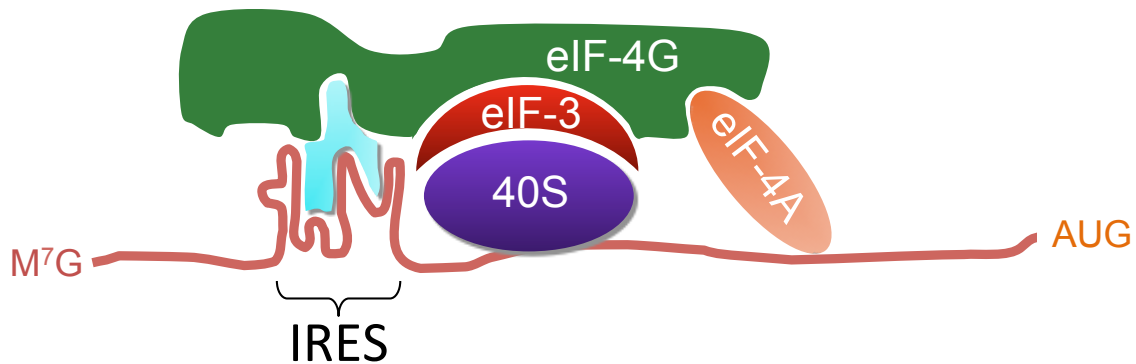
Figure 1.5. **A schematic representation of eukaryotic IRES dependent translation
initiation.** IRES dependent translation utilizes the sequence in the 5' UTR (IRES) to recruit
the 40S ribosome and initiation factors independent of the m7G cap and the eIF3E protein.
Additional factors called ITAFs (blue) bind directly IRES sequences serving as a scaffold for
complex formation, appear to be are required by eukaryotic IRESes.

Of direct relevance to neurodegenerative disease, the APP mRNA was shown

to have upregulated IRES-mediated translation in response to oxidative stress (Beaudoin,

Poirel, & Krushel, 2008). Recently, work done by our lab showed that the tau mRNA

contains an IRES in its 5'UTR. However, how and when this IRES is utilized, and the

mechanisms of its regulation is not known.

Defining the eukaryotic IRES

Unlike viral IRESes, the characteristics that specify eukaryotic sequences are less

well understood and cannot be classified into groups. Cellular IRESes can be relatively short,

as observed for the Gtx mRNA (9 nt)(Chappell, Edelman, & Mauro, 2000), and the APP

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mRNA (50 nt)(Beaudoin et al., 2008; Cencig et al., 2004) or alternatively extremely long as is seen for the voltage-gated potassium channel subunit Kv1.4 mRNA (>1000 nt) (G. M. Jang et al., 2004). The lack of sequence similarity between currently identified cellular IRESes suggests that either they comprise a large group of unique sequence motifs, or that specific structural motifs are commonly recognized. For example, a Y-shaped double hairpin structure is found in many cellular IRESes including the FGF-2 and Bip mRNAs (Le & Maizel, 1997). Another motif postulated for identifying IRES is the presence of sequence complementary to the 18S rRNA as in the case of Gtx, Rbm3, and IGF1R mRNAs (Chappell et al., 2000; Chappell & Mauro, 2003; Meng, Jackson, Shcherbakov, Choi, & Blume, 2010).

Despite the importance of secondary structure in viral IRESes and the identification of clear structural motifs in eukaryotic IRESes, there is mixed opinion about the importance of higher order RNA structure in regulation of cellular IRESes. Our lab and others have shown that secondary structure can play an essential role in IRES activity for specific mRNAs. For example, the 5'UTR of MAPT mRNA exhibits extensive secondary structure. Disruption to this structure by mutation or deletion is detrimental to its IRES activity (Veo & Krushel, 2012). Similarly, the RNA structure present in the Apaf-1 mRNA 5'UTR clearly serves as a binding region for ITAFs required for IRES-mediated translation (Sally A. Mitchell, Spriggs, Coldwell, Jackson, & Willis, 2003). On the other hand, studies done by Holcik lab demonstrates correlation between high eukaryotic IRES activity and weak secondary structure (Xia & Holcik, 2009). Due to the heterogeneity in sequence and structure, there is currently no consensus on defining characteristics of cellular IRESes. As a result all suspected IRES sequences require functional analysis to confirm their ability to mediate cap-independent translation.

Regulation of IRES-dependent translation by signaling pathways

Unlike cap-dependent translation, a generalized pathway serving to regulate all IRES-dependent translation has not been identified. Importantly, IRES-mediated translation is not activated only when cap-dependent translation is inhibited. Instead, there are numerous examples of gene-specific regulation of IRES-mediated translation during key cellular functions such as division, response to proliferative signals, apoptosis, etc. What follows is a summary of major regulatory pathways that have been described to date.

Despite its clear role in regulation of cap-dependent translation, there are examples of involvement of the PIK3-AKT-mTOR pathway in IRES-mediated translation. Oncoproteins cyclin D1 and c-myc are negatively regulated by AKT activity and allosteric mTOR inhibition by rapamycin enhances their IRES dependent translation (Shi, Sharma, Wu, Lichtenstein, & Gera, 2005). Another example is the regulation of LEF1 IRES in chronic myeloid leukemia (CML) that results from the Bcr-abl fusion protein driving tumorigenesis by promoting enhanced translation through the mTOR pathway (Prabhu et al., 2007).

It is important to note, however, that there is probably a crosstalk between multiple signaling pathways, leading to the regulation of a given IRES. Both cyclinD1 and c-myc IRESes are upregulated by rapamycin in a p38 MAPK via MNK, and RAF/MEK/ERK dependent manner, and requires hnRNP A1 as an accessory factor (Jo et al., 2008) (Shi et al., 2013).

ITAFs

Despite the inconsistency in their sequence and structure, all cellular IRES are thought to require accessory factors (termed ITAFs) for their activity. The molecular mechanism of how a given ITAF modulates IRES function varies between IRESes, cellular

environment, and cell types. However, it is speculated that ITAFs bind IRESes and either act to stabilize the IRES structure and facilitate binding of initiation factors (Figure 1.5) or are somehow able to directly promote ribosome recruitment (King, Cobbold, & Willis, 2010; Spriggs, Bushell, Mitchell, & Willis, 2005). Alternatively, binding by some ITAFs act to inhibit IRES dependent translation. In such instance, these inhibitory ITAFs either compete with positive ITAFs for binding, or they block binding of translational factors, and ribosome. Thus ITAF-mediated translation is both positively and negatively regulated.

Most of the ITAFs identified to date are heterogeneous nuclear ribonuceloproteins (hnRNPs), which are known to be involved in RNA processing. La autoantigen, which is another RNA binding protein, has been shown to stimulate the XIAP IRES and aid in binding of 40S ribosomal subunit (Holcik & Korneluk, 2000). Other ITAFs include the eIF4G family member eIF4G2 (also called p97/DAP5), which shares homology with the C-terminus of the scaffolding protein eIF4G1 such that it is unable to bind eIF4E (Imataka, Olsen, & Sonenberg, 1997). EIF4G2 promotes IRES-dependent translation of several IRESes including the XIAP, and HIAP2 IRES during programmed cell death or ER induced stress (Lewis et al., 2008; Nevins, Harder, Korneluk, & Holcik, 2003).

One mechanism by which differential signaling may affect IRES dependent translation of select mRNAs is via the PTM of specific ITAFs. PTMs have been demonstrated to regulate expression levels of ITAFs, subcellular localization of ITAFs (nuclear vs cytoplasmic) and binding of ITAFs to IRESes. Phosphorylation of ITAFs is the most studied out of all PTMs. Many ITAFs are phosphorylated by different upstream kinases under different physiological conditions.

One of the ITAFs whose phosphorylation is most studied is hnRNPA1. HnRNPA1 is

a well known ITAF for XIAP (Lewis et al., 2007), BCL-XL, Apaf-1 (Cammas et al., 2007), cyclin D1, c-myc (Jo et al., 2008), SREBP-1a (sterol-regulatory-element binding protein 1(in ER stress)(Damiano et al., 2013), EGR2 (Rubsamen et al., 2012) and FGF2 (Bonnal et al., 2005). There are also examples showcasing an Akt-independent role of mTORC1 complex in IRES mediated translation. During G2/M phase of the cell cycle, upstream PI3K and Ras/Erk pathways are downregulated. However raptor protein in the mTORC1 complex is phosphorylated at novel sites via the cyclin dependent kinase (CDK1) and GSK3 activity. It was shown that this novel phosphorylation event and mTORC1 activity is required for enhancement of rapamycin resistant EMCV IRES activity during G2/M (Ramirez-Valle, Badura, Braunstein, Narasimhan, & Schneider, 2010). IRES dependent translation of several mRNAs (including the G2-M kinase p58PITSLRE, c-myc, ornithine decarboxylase (ODC), the p53 tumor suppressor, cyclin dependent kinase inhibitor p27) is upregulated during mitosis (Cornelis et al., 2000; Miskimins, Wang, Hawkinson, & Miskimins, 2001; Pyronnet et al., 2000; Ray, Grover, & Das, 2006).

1.3 Polypyrimidine tract binding protein (PTB)

PTB, also known as hnRNPI, is a 57 KDa RNA-binding protein that binds polypyrimidine-rich (C and U nucleotides) regions of RNA. PTB protein consists of four highly conserved RNA recognition motifs (RRM) that allows for its sequence-specific high affinity binding. The PTB gene is located in chromosome 19p13.1, and consists of 15 exons, which undergo alternative splicing to produce four different isoforms PTB1, PTB2, PTB3, and PTB4 (Romanelli, Diani, & Lievens, 2013; Sawicka, Bushell, Spriggs, & Willis, 2008).

There are two other proteins - neural PTB (nPTB, or PTBP2), and regulator of differentiation 1 (ROD1) – that share 70-80% homology to PTB. The nPTB protein is

expressed mainly in the neurons in the brain and the retina, but its expression is also seen in other tissues such as myoblasts, lymphocytes, and testis (Boutz et al., 2007; Cheung et al., 2009). The peptide sequence of nPTB shares 74% homology to PTB with near identical sequences in their RNA binding motifs (Keppetipola, Sharma, Li, & Black, 2012; Markovtsov et al., 2000). The RNA binding properties of PTB and nPTB are very similar, with one lysine to arginine and one phenylalanine to tyrosine change in their RNA binding regions, but their binding affinity to the RNA vary (Markovtsov et al., 2000).

Role of PTB in splicing

PTB is a multifunctional protein with roles in mRNA splicing, localization, transport, polyadenylation, stability, and translation (as an ITAF). These various functions implicate PTB in development and diseases. The role of PTB as a negative regulator of alternative splicing has been well documented. As a splicing repressor, PTB is known to suppress exon inclusion of many RNAs including IgM, Fas, FGFR2, c-src, USP5 and many more (Chan & Black, 1997; Izaguirre et al., 2012; Sawicka et al., 2008; Tollervey et al., 2011). Interestingly, PTB auto-regulates its expression, and regulates the expression of nPTB via alternative splicing. During elevated levels of PTB expression, PTB binds to its own mRNA near the 3' end of exon 11. This causes skipping of the exon leading to nonsense-mediated decay (NMD) of the RNA and a concomitant reduction in PTB protein levels (Wollerton, Gooding, Wagner, Garcia-Blanco, & Smith, 2004). Similarly, PTB suppresses exon 10 of nPTB leading to NMD of nPTB mRNA, thereby limiting nPTB expression in cells where PTB is expressed (Boutz et al., 2007).

In the nervous system, PTB is expressed in neural progenitor cells. However, during neuronal differentiation, brain specific microRNA (miR), miR-124 targets PTB and down

regulates its expression (Makeyev, Zhang, Carrasco, & Maniatis, 2007).. This reduction in

PTB is postulated to modulate nPTB transcript splicing such that protein production is

upregulated (Boutz et al., 2007; Makeyev et al., 2007; Spellman, Llorian, & Smith, 2007).

This reduction in PTB protein and upregulation of nPTB protein fosters a neuron-specific

pattern of precursor mRNA splicing that is associated with cellular differentiation. Thus, in

the adult brain, PTB is expressed in non-neuronal cells (such as glia), while nPTB is

expressed in mature neurons (Boutz et al., 2007). PTB mediated alternative splicing is further

upregulated during aging and in neurodegenerative diseases, while the expression of nPTB,

and neuro-oncological ventral antigen (NOVA) proteins as well as NOVA-dependent

splicing is decreased (Tollervey et al., 2011).

Role of PTB in RNA transport and stability

There are many examples demonstrating the role of PTB in RNA processing

including polyadenylation, localization, and stability. During neurite growth, PTB is

localized to the neurite terminals with α-actin, and mutation of PTB binding site in the α-

actin 3'UTR hinders this localization and prevents neurite growth (S. Ma, Liu, Sun, & Xie,

2007). Besides implicating PTB in neurite growth, this study showed that PTB-mediated

localization is important for regulation of translation in a spatial and temporal manner. The

role of PTB in mRNA stability is also seen in preproinsulin mRNA, vascular endothelial

growth factor (VEGF), hypoxia inducible factor (HIF-1α), nitric oxide synthase, activated T

and B cells, and other transcripts (Romanelli et al., 2013; Sawicka et al., 2008). PTB binding

to CU-rich sequences in the 5' UTR, or 3'UTR of mRNAs have additional roles in 5'- and

3'-end processing. PTB binding increases the 5'- end processing of complement 2,

protrombin, and cyclooxygenase-2 pre-mRNAs, while it decreases the 3'end cleavage of α-

globin and β-globin RNAs. In addition, PTB binding to certain mRNAs such as the 3'UTR of the mper2 mouse circadian clock leads to mRNA decay. More recently, studies have shown that PTB binding to mRNAs may also repress translation by impeding binding by the translational machinery. This is seen in the case of *Drosophila oskar* mRNA, where such regulation by PTB shows its importance in embryonic development (Romanelli et al., 2013).

Role of PTB as an ITAF

Although PTB has extensively studied roles in RNA metabolism, it has equally important roles in regulation of translation. It is these roles that are of direct relevance to my thesis studies. PTB was one of the earliest factors identified to serve as an ITAF for many viral and cellular IRESes (Borovjagin et al., 1994) (Cho, Kim, Back, & Jang, 2005; Cornelis, Tinton, Schepens, Bruynooghe, & Beyaert, 2005; Dhar, Venkataramana, Ponnuswamy, & Das, 2009; Holcik & Korneluk, 2000). IRESes require specific structural conformation of the RNA to allow binding of the ribosome or other factors of the translational machinery. One mechanism by which PTB promotes IRES mediated translation is by binding to the IRES and stabilizing the IRES structure to facilitate ribosome recruitment. This is seen for EMCV, PV, and TMEV IRESes. PTB is also able to bind multiple pyrimidine stretches within the IRES thereby bridging the sites to provide a suitable conformation for ribosome recruitment. This RNA chaperone function of PTB is seen in picornaviral IRESes. In addition to viral IRESes, a number of studies have documented PTB's role as an ITAF in many cellular IRESes. In eukaryotic cells, IRES-dependent translation is thought to occur in conditions of cellular stress such as hypoxia, apoptosis, inflammation, and mitosis. PTB has been shown to be required for the translation of specific IRESes under these conditions of cellular stress. For example, PTB binds to the 5'UTR of insulin mRNA to regulate insulin biosynthesis during

conditions of nitrosative stress (Fred, Bang-Berthelsen, Mandrup-Poulsen, Grunnet, & Welsh, 2010; Fred, Sandberg, Pelletier, & Welsh, 2011). During cell-cycle, PTB modulates the IRES dependent translation of cyclin dependent kinase 11 (CDK11) (Ohno, Shibayama, Sato, Tokunaga, & Yoshida, 2011), while during inflammation, PTB functions to enhance early growth response 2 (egr2) IRES dependent translation (Rubsamen et al., 2012). PTB also regulates cationic amino acid transporter 1 (cat-1) IRES during starvation, while PTB translocates p53 mRNA from nucleus to cytoplasm to allow p53 IRES dependent translation during stress (Grover, Ray, & Das, 2014).

Some IRESes require additional ITAFs along with PTB for their translation. One such example is the Bcl-2 associated athanogene 1 (BAG-1) IRES, which requires another ITAF poly (rC) binding protein (PCBP1) binding to change the RNA conformation such that PTB can bind and allow ribosomal recruitment (Pickering, Mitchell, Spriggs, Stoneley, & Willis, 2004). In the case of Apaf-1 IRES, UNR first binds to the IRES causing a conformational change to allow PTB binding for its translation (Sally A. Mitchell et al., 2003). While most of these examples show PTB binding to the 5'UTR of mRNA to modulate IRES dependent translation, PTB has also been shown to activate IRES through binding to the 3' UTR as in the case of hypoxia inducible factor 1-α (HIF1-α) IRES. PTB binds to the 3' UTR of HIF1-α mRNA and promotes the 3' UTR -5'UTR interaction via its interaction with another ITAF HuR which binds the 5' UTR (Galban et al., 2008). While PTB has been shown to promote IRES activity for most IRESes, there are few IRESes whose IRES activity are suppressed by PTB. IRES dependent translation of Bip and UNR IRESes are inhibited by PTB (Cornelis et al., 2005; Kim, Hahm, & Jang, 2000).

One explanation of how PTB is able to function in these various ways is through its

ability to bind to a multitude of CU-rich stretches via the specificity of its RRMs. While the RRM1 and RRM2 are known to bind short CU-stretches in stem loops, RRM3 and RRM4 bind longer CU-stretches of single stranded RNA (Clerte & Hall, 2009). The exact PTB consensus site has been a subject of debate since PTB has been known to bind UCUU, (CCU)n, UCUUC, UCUUU or CUCU sequences (S. A. Mitchell et al., 2005). In addition, work done by the Willis lab postulated (CCU)n as an IRES motif required by all cellular IRESes. They showed that the presence of a artificial (CCU)n hairpins is sufficient to drive internal initiation (S. A. Mitchell et al., 2005). This data suggests that PTB not only binds to IRESes and change RNA conformation, but it also acts as a bridge between the IRES and the ribosome. PTB function on IRESes is also dependent upon the cell type as well as cellular conditions, all of which regulate availability of PTB or its binding partners.

1.4 Scope of the study

The regulation of tau protein by transcription, phosphorylation, and alternative splicing has been extensively studied. In contrast, the mechanisms of translational regulation of tau expression are not known. Deregulating tau expression contributes to neurodegenerative disease including Alzheimer's disease and other tauopathies. Work done in our lab has previously demonstrated that tau mRNA contains an IRES. I hypothesize that the tau IRES is highly regulated and contributes to the regulation of tau protein expression. In this study, I focused on understanding the regulation of internal translation mediated by the tau IRES by -

1) examining cellular conditions, and signaling pathways that regulate the tau IRES,

2) identifying ITAFs involved in tau IRES mediated translation.

Chapter II

Materials and Methods

2.1 Constructs and cloning

All constructs were generated using standard established cloning methodology. For the most part constructs fall into two major classes, plasmids used for the generation of *in vitro* transcribed RNA and plasmids containing eukaryotic promoters for in vivo expression of RNA. A list of primers used for cloning and their sequences is provided in Table 2.1.

The monocistronic luciferase constructs are based on previously described reporter plasmids (Veo & Krushel, 2009). Briefly, the *Photinus* gene and SV40 3' UTR from the dicistronic RP vector (Figure 2.1 A) (Stoneley et al., 2000) were cloned into PBluescript II SK (+) (pBS) downstream of the T7 promoter for *in vitro* transcription using EcoRI and BamHI restriction sites. The 5' UTRs of tau, APP, β-globin, EMCV, or AurA were amplified by PCR and inserted upstream of *Photinus* sequence in the monocistronic pBS using EcoRI and NcoI sites. The design of primers and restriction sites was made to allow placement of 5'UTR sequence in the same relative position to the *Photinus* translation start as that of each leader sequences naturally occurring AUG start site. The resulting vectors were called tau-MBS, APP-MBS, β-globin-MBS, EMCV-MBS, and AurA-MBS (Figure 2.1 B) (Beaudoin et al., 2008; Veo & Krushel, 2009) (Dobson, Chen, & Krushel, 2013). The *Renilla* luciferase construct used to normalize transfections has been previously described (Dobson et al., 2013; Veo & Krushel, 2009).

For RIP experiments requiring in vivo transcription, tau-MBS, APP-MBS and β-globin-MBS constructions were digested using EcoRI and BamHI restriction endonucleases to generate a fragment containing the leader upstream of *Photinus* luciferase sequence and SV40 poly A sequence. The resulting 5'UTR-containing fragments were gel purified and

cloned into pcDNA3.1 vector, which contains a CMV promoter and SV40 polyA sequences (Figure 2.1 C).
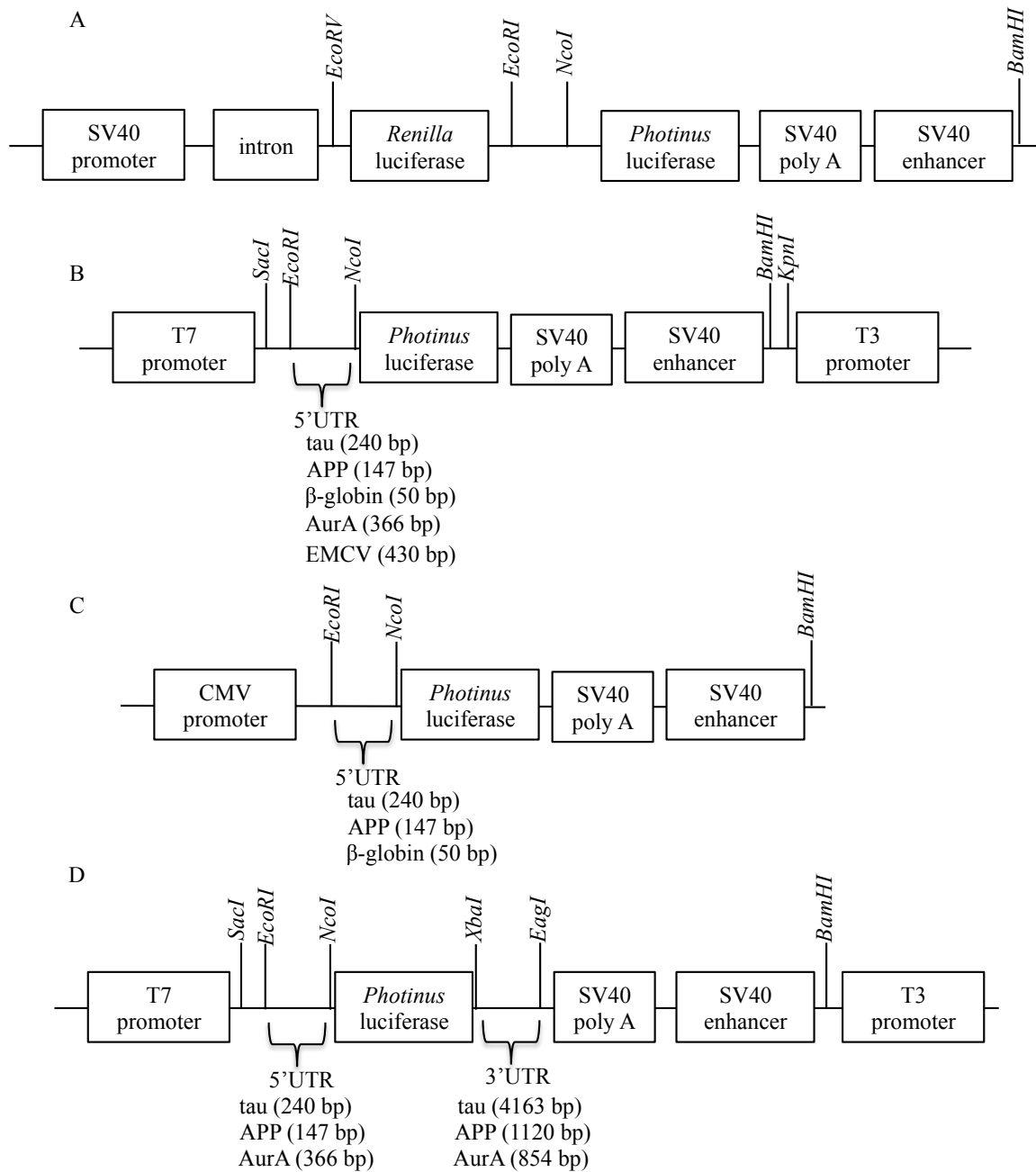
Figure 2.1. **Schematic map of constructs used**.

The generation of the monocistronic constructs containing their respective 3' UTRs required a multistep process. The human tau 3' UTR, APP 3'UTR, or AurA 3'UTR sequences were amplified by PCR using primers containing XbaI and EagI restriction sites and TA cloned in a TOPO vector (Thermofisher). An existing EagI restriction site within the tau 3' UTR was mutated using QuikXL mutagenesis kits using tau 3'UTR EagI mut forward and reverse primers. Duplicate XbaI and EagI within the multilinker near the poly A site in the pBS vector were also mutated such that only one set of the restriction sites remained. The TOPO vectors containing the 3'UTRs were XbaI/EagI digested to generate fragments that were gel purified for insertional cloning into the pBS vectors with the respective 5'UTRs. Additionally, the tau 3'UTR was cloned into APP-MBS, and AurA-MBS as well. All constructs were then mutated again to restore the original sequence with the EagI site (Figure 2.1D).

For tau 3'UTR deletion studies, a cloning strategy similar to that for the full length 3'UTR was used. Forward primers containing XbaI site and reverse primers containing EagI site were used to amplify the first 1000 bp, 3000 bp, 3750 bp or 4000 bp, or the last 500 bp, or 163 bp of the tau 3' UTR. The resulting sequences were cloned into tau-MBS downstream of the *Photinus* sequence using XbaI and EagI sites.

Constructs containing targeted mutations of the putative PTB binding sites in the tau 5'UTR (Fig 5) were generated by QuikXL mutagenesis. Tau-MBS construct was used as a template with targeted mutations introduced using the following forward and reverse primers- PTBmut1, PTBmut2, and PTBmut3. All constructs were verified by sequencing at MD Anderson core sequencing facility.

Table 2.1. **List of sequencing primers used in the study.**

Primer name	Sequence
Tau3'UTR1 XbaIFwd	TCATTCTAGATCAGGCCCCTGGGGCGGT
Tau3'UTR3000 BamH1Rev	ATGGATCCAGAGGAACCGAGGTGC
tau3'UTR2000 BamH1Rev	ATGGATCCGTGAGAGACACCTCGTGA
Tau3'UTR1000 BamH1Rev	ATGGATCCTAGCCCTAAAGTCCCA
Tau3'UTRXbaI mutFwd	GAATTGCCTGTCCTGGATCTGCTTTAGAGGCCCAAGCTGC
Tau3'UTRXbaI mutRev	GCAGCTTGGGCCTCTAAAGCAGATCCAGGACAGGCAATTC
Tau3'UTRXbaI mutbackFwd	GAATTGCCTGTCCTGGATCTGCTCTAGAGGCCCAAGCTGC
Tau3'UTRXbaImut backRev	GCAGCTTGGGCCTCTAGAGCAGATCCAGGACAGGCAATTC
APP3-XbaIF	AGATCTAGACCCCCGCCACAGCAGCCT
APP3-HpaIR	GGCGGTTAACTGCTCCTCCAAGAATGTAT
APP3-520HpaIR	CGGCGGTTAACGGAAAAAAAATCTCTCTA
Tau3-3000HpaIR	ATGTTAACAGAGGAACCGAGGTGC
Tau3-2000Hpa1Rev	ATGTTAACGTGAGAGACACCTCGTGA
Tau3-1000Hpa1Rev	ATGTTAACTAGCCCTAAAGTCCCA
APP3-EagIR	GGCGCGGCCGTGCTCCTCCAAGAATGTAT
APP3-520Eag1R	CGGCGCGGCCGGGAAAAAAAATCTCTCTA
Tau3-3000EagIR	ATCGGCCGAGAGGAACCGAGGTGC
Tau3-2000Eag1Rev	ATCGGCCGGTGAGAGACACCTCGTGA
Tau3-1000Eag1Rev	ATCGGCCGTAGCCCTAAAGTCCCA
Tau3-4000Eag1Rev	CATCGGCCGTTTAATCAGAGTAATAACT
Tau3-XbaIFwd	ATAATCTAGATCAGGCCCCTGGGGCGG
AurA3-XbaIFwd	CCGATTCTAGAGTACTTGAACACCCCTG
AurA3-EagIRev	CGCGGCCGAGACATAGATACTTAT
AurA3-425EagIRev	CGGCCGATTTGCCTCCTGTGAA
pBSXbaEag mutFwd	AGCGGATCCACTAGTACTAGAGAGGCCGCCACCGCGG

pBSXbaEag mutRev	CCGCGGTGGCGGCCTCTCTAGTACTAGTGGATCCGCT
Tau3-EagmutFwd	TATGCCACCTGCAGCGTCTGAGAGGCCGCCTGTCCTTGGT
Tau3-EagmutRev	ACCAAGGACAGGCGGCCTCTCAGACGCTGCAGGTGGCATA
AurA3-xbamutFwd	CCATTGACTGCTGCCCTCAACCTAGAACGCTACACAAG
AurA3-XbamutRev	CTTGTGTAGCGTTCTAGGTTGAGGGCAGCAGTCAATGG
TauPTBmut1Fwd	GCTTGATATCGAATTCGGAGGGGAGGGGAGGCTCGCGTTC
TauPTBmut1Rev	GAACGCGAGCCTCCCCTCCCCTCCGAATTCGATATCAAGC
TauPTBmut2Fwd	CTCAGGAACGCGGAAAGAAGGCCGGCGCGCGCCCT
TauPTBmut2Rev	AGGGCGCGCGCCGGCCTTCTTTCCGCGTTCCTGAG
TauPTBmut3Fwd	CCACCACAGCCAGGTTGTGGTGGTGGGCTGTCCTCTC
TauPTBmut3Rev	GAGAGGACAGCCCACCACCACAACCTGGCTGTGGTGG
AurAXbaIFwd	ACTTCTAGACAAGGCAGCCTCGCTCG
AurAEagIRev	CGGCCGATGCCTGTAATCCCAGCT A
tau3750EagIRev	CGGCCGCTCAGCTCCAAAAGCACA
Tau4001EagIRev	CGGCCGATCATGGGACTTGCAAGT G
Tau250Eag1Rev	CGGCCGATTACTAGCCCACCCATCA
Tau500Eag1Rev	CGGCCGCTCACACAAGGTTGACAT
Tau780Eag1Rev	CGGCCGCAGCTTGCCTTCTCTT
Tau3730-4163mutFwd	GTGTATTGTGTGTTTTAACAAATGATTTACACTCTGACAAGC TGTAAAAGTGAATTTGG
Tau3730-4163mutRev	CCAAATTCACTTTTACAGCTTGTCAGAGTGTAAATCATTTGT TAAAACACACAATACAC

2.2 Cell Culture

All cell culture was maintained using standard accepted methodology and sterile

technique. The SKNSH cell line was obtained from ATCC and maintained in Modified

Eagle's Media (MEM), with 10% fetal bovine serum and 1% pencillin/streptomycin. The

U87 and Daoy cell lines were generously provided by Dr. Vidya Gopalakrishnan (UT MD Anderson Cancer Center) and the rat C6 glioma cell line was obtained from ATCC. All three cell lines were cultured in DMEM, with 10% fetal bovine serum, and 1% pencillin/streptomycin. The MCF12A cell line was provided by Dr. Heide Ford, and cultured as previously described (Ford, Kabingu, Bump, Mutter, & Pardee, 1998).

2.3 *In vitro* transcription

The monocistronic pBS-based constructs were linearized with BamHI (tau-MBS, EMCV-MBS, or β-globin-MBS) , or NotI (APP-MBS) to generate template for transcription. The linearized plasmid was gel purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research). For each assay 1μg of linearized plasmid DNA was transcribed at 37°C for 3 hours using MEGAScript® T7 kit (Ambion). The ApppG capped messages were produced by the inclusion of 3 μL 40 mM G(5')ppp(5')A RNA cap analog and 2 μL 15mM GTP to the transcription reactions. For transcripts containing a standard cap, m7G was added using Script Cap™ m7G capping system (Cellscript.Inc). The transcription reactions were then treated with 1μL DNase (2 U/μL) for 15 mins at 37°C. The mRNA was then purified using phenol/chloroform and isopropanol precipitation, dried, and resuspended in nuclease free water. A poly (A) tail was added to all transcripts using Poly (A) Polymerase tailing kit (Epicentre) per manufacturer's instructions followed by phenol/chloroform extraction, isopropanol precipitated, and finally resuspended in nuclease free water. RNA size and quality was verified by running 1 μg of poly(A) transcript on a Reliant® Precast RNA gel (LONZA) for 1.5 hours and visualized by SYBR® gold staining (Invitrogen).

2.4 Cell treatments

All cell line treatments were performed on subconfluent cells. Cells were plated at 2

X 10^6 per well of a 12-well dish and treatment was added with a change of medium after 24 hours.

A. Chemical ischemia: The SKNSH cell line was treated with an acute 10 minutes exposure to 10 mM sodium azide and 10 mM 2-deoxyglucose followed by RNA transfection.

B. FAC treatment: The SKNSH cell line was treated with ferric ammonium citrate (FAC) supplemented media for 24 hours before RNA transfection.

C. Aβ (1-42) peptide treatment: The SKNSH cell line was exposed to10 μM of human amyloid β-peptide (1-42) (Tocris) for 16 hours before RNA transfection.

D. Poly (I:C) treatment: The SKNSH cell line was exposed to 500 μg/ml of poly I:C for 30 minutes after RNA transfections.

E. PP242 treatment and puromycin incorporation: The Daoy cell line was treated either with DMSO (vehicle) or 2.5 μM PP242 for two and half hours. The growth medium was then supplemented with 1 μM puromycin (Invivogen) for thirty minutes to terminate protein synthesis. Cells were then harvested and puromycin incorporated nascent polypeptides were detected by western blotting using puromycin antibody.

F. Growth factors: The Daoy cell line was serum starved for 4 hours prior to the addition of the following growth factors: 10nM EGF, 100nM insulin like growth factor-1 (IGF-1), 100nM insulin, 20 ng/mL basic fibroblast growth factor (bFGF), 100 ng/mL brain-derived neurotrophic factor (BDNF), 100 ng/mL GDNF, 5 ng/mL Hrg-1β, 100 ng/mL artemin, or 10 ng/mL neurturin. Cells were then transfected with RNA for four hours.

G. Inhibitors: The Daoy cell line was treated with the following inhibitors for a total of seven hours:10 μM U0126 (MEK inhibitor, Millipore), 20 μM LY294002 (PI3K inhibitor, Sigma), 10 μM Akt inhibitor III (Calbiochem), 10 μM SP600125 (JNK inhibitor, company),

10 μM SB203580 (p38 MAPK inhibitor, Tocris), 2.5 μM PP242 (mTOR inhibitor, Tocris), 5

μM BRD7389 (RSK inhibitor, Sigma), 10 μM MNK1/2 inhibitor (Calbiochem), 10 μM DG2

(S6K inhibitor, Calbiochem), and 50 μM AR-A014418 (GSK3β inhibitor, Sigma). Following

three hours of inhibitor exposure cells were transfected with RNA and lysates/RNA

harvested after four hours. A list of inhibitors used, their targets, and IC50 is provided in

Table 2.2, and a schematic of signaling molecules that these inhibitors target is provided in

Figure 2.2.
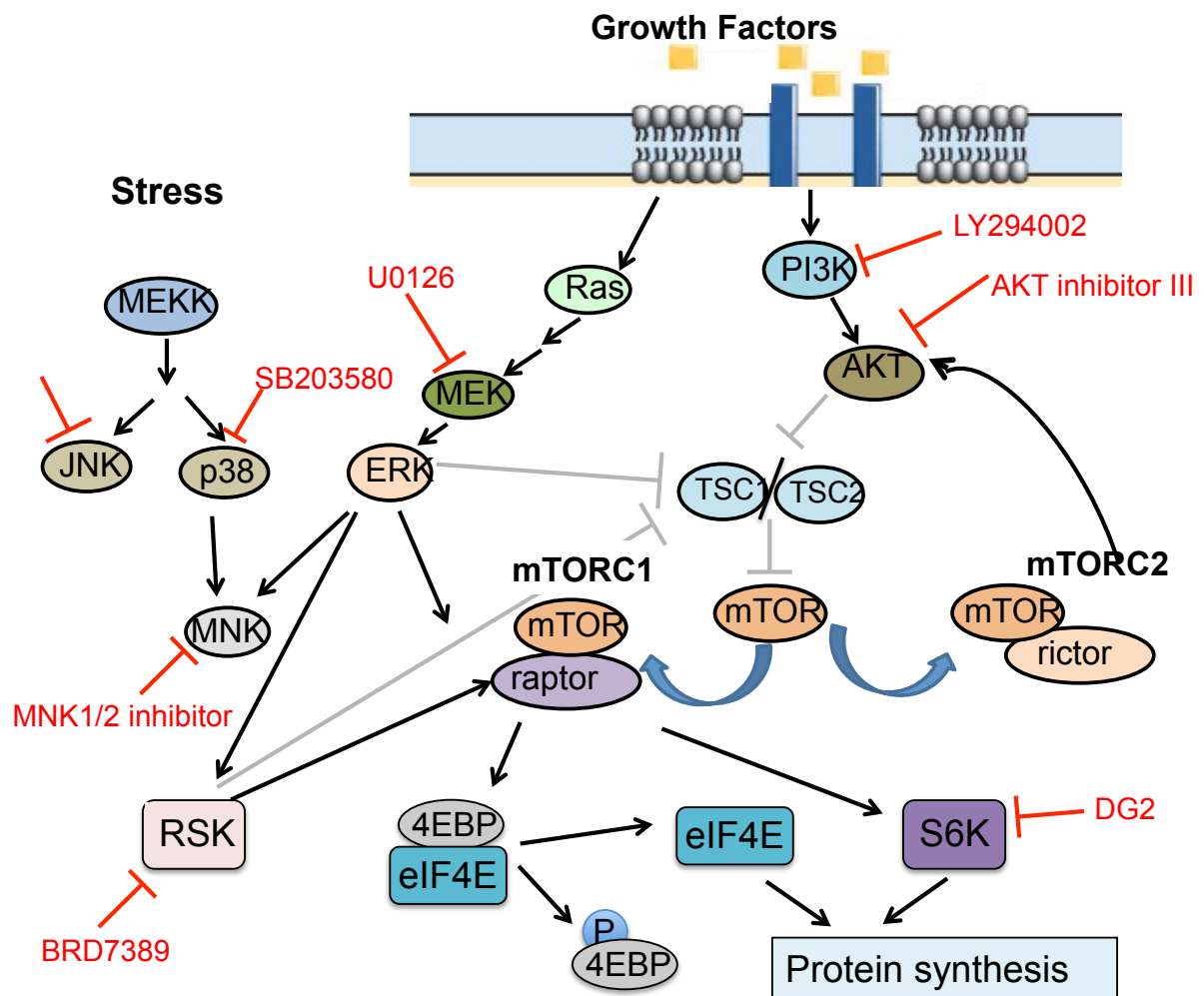
Figure 2.2. **Schematic representation of interacting pathways that regulate protein synthesis and pharmacological inhibitors targeting them (shown in red).**

Table 2.2. **List of inhibitors used in the study.**

Inhibitor	Target	IC50
PP242	mTORC1/mTORC2	8 nM
SB203580	p38MAPK	50-500 nM
SP600125	JNK	40-90 nM
U0126	MEK1/MEK2	0.06- 0.07 µM
LY294002	PI3K	0.31-6.6 µM
AKT inhibitor III	Akt	5-10 µM
BRD7389	RSK	1.5 µM
MNK 1/2 inhibitor	MNK1/ MNK2	575-646 nM
DG2	S6K	9.1 nM
AR-A014418	GSK3	104 nM

2.5 *In vitro* Translation

Rabbit reticulocyte lysate (RRL)(Promega) was used to perform *in vitro* translations. Reactions were assembled as per manufacture protocol with 17.5 µL RRL, 0.25 µL Amino acid mixture –Leu, 0.25 µL Amino Acid mixture –Met, 0.5 µL RNase inhibitor, and 5 µg of *in vitro* transcribed A-capped mRNA. Translation reactions were performed using incubated for 1 hour at 30°C. The sample was then immediately assayed for *Photinus* and *Renilla* luciferase activity.

In experiments using depleted RRL, target proteins were removed using an antibody conjugated bead approach. Specific protein targets were recognized using the following antibodies: rabbit anti-eIF4G1 (Abcam), mouse anti-PTB (Invitrogen), mouse anti-La (BD Biosciences), or rabbit anti-IgG (Cell signaling). Antibody's were adhered to protein G coated magnetic beads overnight at 4°C. Antibody conjugated magnetic beads were washed 3 times using excess wash buffer, then added to 100 µl of RRL, and incubated at 4°C for one

hour. RRL and the beads mixture were separated using a magnet to generate the depleted RRL used in translation assays.

In experiments using supplemented RRL exogenous factor addition, 0.0001-1 μM of recombinant nPTB or GST (Genecopoeia) was added to the translation reaction.

2.6 Dual luciferase assay

In all cases luciferase measure was performed using the Dual-Luciferase Reporter Assay (DLA) System (Promega) and analyzed using a Luminoskan luminometer. Cells were lysed for 15 minutes at room temperature using 250 μl 1X passive lysis buffer (Promega) for one well of a 12-well plate. Our standard assay used 40 μl of lysate from transfected cells, or *in vitro* translation assays.

2.7 Lentiviral Transduction

The coding sequences of PTB or nPTB ORF (kindly provided by Dr. Chris Smith, University of Cambridge) were excised and introduced into a HIV-lentiviral expression vector pTRH1-mCMV. HIV-lentiviruses were created by Dr. Jerry Schaack in the viral vector core (University of Colorado Denver School of Medicine) and provided at a titer of 3.7X107 pfu/mL. Lentiviral infection was conducted in U87 cells. Cells were plated at 50% confluency, and infected using a Polybrene® solution (Millipore) at 4 μg/mL in 2 mL of complete media at a multiplicity of infection (MOI) of 5. Cells were incubated for 6 hours, after which the media was aspirated off and replaced with fresh complete media. Cells were then incubated for 48, or 72 hours to identify the optimal protein expression. A RFP viral control (a generous gift from Dr. Li Ma, MD Anderson Cancer Center) was also used to verify that the cells could be infected. Cells were then harvested for western blotting (as described).

2.8 siRNA treatment

Two different protocols were used for siRNA-mediated knockdown of target proteins.

Transfection was dependent upon the recommendations of the manufacturer of specific

siRNAs. Targeting PTB, nPTB, or La was performed using endoribonuclease prepared

siRNA pools (MISSION esiRNA, Sigma). EGFP esiRNA was used as a control. For these

esiRNA pools Lipofectamine RNAiMAX reagent (Thermofisher) was used for transfection.

Knockdown for Raptor, Rictor, PCBP2, PRKX, ULK2 proteins, three different siRNAs

(Sigma) targeting each gene were used. Sigma siRNA reagent was used for transfection. To

examine the impact of specific knockdown on translation cells were exposed to siRNA for

48-72 hours prior to a second transfection with *in vitro* transcribed RNAs. Protein lysates

were generated for luciferase assay. Efficiency of all knockdowns was examined by western

analysis.

2.9 Western Blot

Immunoblot analyses were performed using established methodology. In all cases cell

lysates were prepared by scraping cells on ice in lysis buffer (Promega) containing protease

(Roche) and phosphatase inhibitors (Pierce). Lysates were centrifuged for 20 minutes at

12000 rpm at 4°C to remove cellular debris. Protein content of the lysates was quantitated

using Bradford assay. Lysate samples (15-25 µg) were denatured by boiling for 5 minutes in

SDS loading dye (20% glycerol, 4% SDS, 0.2 M Tris pH 6.8, 200 mM DTT, 0.2%

bromophenol blue) and loaded onto a 10% SDS-PAGE gel, and run at 200 volts for 45

minutes in a Biorad Mini-PROTEAN electrophoresis chamber using running buffer (1X Tris-

glycine buffer, Biorad, 10% SDS). Proteins were transferred onto nitrocellulose membrane

for 45 minutes at 400 mA using cold transfer buffer (Tris glycine and 20% methanol). The

same Biorad electrophoresis chamber was placed on ice and used for the transfer. 5µl of

Precision Plus Protein Western Blotting standards (Biorad) was used as for protein ladder.

After transfer, the blots were blocked with 5% milk in 1X TBST buffer (0.9% sodium

chloride, 20 mM Tris-HCl, 0.05% Tween-20, pH 7.5) for 1hour at room temperature. The

blots were probed with antibodies diluted in TBST overnight at 4°C. On day two, the blots

were washed with TBST for 10 minutes, this wash was repeated 3 times. A list of antibodies

used in this study is provided in Table 2.3. The blots were then probed with secondary

antibody diluted in TBST for 1 hour at room temperature. The blots were rewashed 3 times

for 10 minutes in TBST. Blots were developed using ECL-plus chemiluminescent detection

reagent (Promega) and detected using clear blue x-ray film (Thermofisher).

Table 2.3. **List of antibodies used in the study.**

Antibodies	Dilution	Source	Catalog #
4EBP1 (53H11)	1:1000	Cell signaling	9644
APP	1:500	Cell signaling	2452
eEF2K	1:1000	3692 Cell Signaling	3692
eIF4G1	1:1000	Cell signaling	2858
GAPDH	1:200	Santa Cruz Biotechnology	FL-335
HRP-conjugated anti-mouse	1:3000	Promega	W4021
HRP-conjugated anti-rabbit	1:20000	Promega	W4011
La	1:1000	BD Transduction	610904
La	1:200	Santa Cruz Biotechnology	sc33593
nPTB	1:200	Santa Cruz Biotechnology	sc98491
nPTB	1:1000	Abnova	H00058155
p-4EBP1(Thr37/46)	1:1000	Cell Signaling	9459
p-eIF4E (Ser209)	1:1000	Cell signaling	9741
p-eIF4G1 (Ser 1108)	1:1000	Cell signaling	2441
p-p70S6K	1:1000	Cell Signaling	2708
P70S6K	1:1000	Cell Signaling	2708
PCBP2	1:!000	Abcam	ab154245
PRKX	1:1000	Abcam	ab109389
PTBP1	1:1000	Thermofisher	324800
puromycin	1:2000	Sigma Aldrich	MABE343
Raptor	1:200	Santa Cruz Biotechnology	sc-81537
Rictor	1:200	Santa Cruz Biotechnology	sc130865
Tau5	1:1000	Thermofisher	AHB4002
ULK2	1:1000	Abcam	ab97695
UNR	1:200	Santa Cruz Biotechnology	79292

2.10 Polysome Analysis

Daoy cells from ten 10-cm plates were pooled for each experimental set (control, or PP242

treated). To stall translation cells were treated with cycloheximide (50 ng/mL) for thirty

minutes prior to polysome isolation. Cells were then harvested on ice in PBS containing 50

ng/mL of cycloheximide and lysed using 400 μl lysis buffer (100 mM KCl, 50 mM TrisHCl,

1.5 mM MgCl$_2$, 1 mM DTT, 1.5% NP-40), containing protease inhibitors (Roche), 100

μg/mL cycloheximide and 100 U RNasin® plus RNase inhibitor (Promega). Polysome

profiling was performed using 300 μl of lysate loaded on a 20–60% sucrose gradient that was

centrifuged at 39,000 rpm for 2 hours at 4 °C using a SW40Ti rotor (Beckman Coulter).

Following centrifugation 10 fractions (1.5 mL each) were collected for analysis.

2.11 RNA extraction and Quantitative Reverse Transcription-PCR

In all cases total RNA was extracted from cells using TRIzol® Reagent (Sigma) followed by

additional purification using the PureLink™ RNA Mini Kit (Invitrogen). Equal amounts of

RNA (0.5 μg) were used to prepare cDNA using iScript™cDNA Synthesis Kit (Bio-Rad).

For RIP experiments, PCR was performed using HotStarTaq DNA Polymerase (Qiagen)

using *Photinus* or GAPDH primers (see Table 2.4). For all other experiments, qRT-PCR was

performed using a Roche Lightcycler® 480 with Advanced™ SYBR® Green Supermix

(Bio-Rad) as per manufacturer's instructions. Triplicates of all samples were used for qRT-

PCR. RNA concentrations were calculated from standard curves for the respective RNAs.

Table 2.4. **List of primers used for qRT -PCR.**

Primer name	Sequence
PhotinusFwd	AAAGCTCCCAATCATCCAAA
PhotinusRev	CAGATGTGACGAACCTGT
GAPDHFwd	ACAGTCAGCCGCATCTTCTT
GAPDHRev	GTTAAAAGCAGCCCTGGTGA
TauRTFwd	ACAAACCAGTTGACCTGAGCA
TauRTRev	GGACCCAATCTTCGACTGGA

2.12 RNA immunoprecipitation assay

Plasmids (pCMV) containing tau, APP, or β-globin leader upstream of *Photinus* luciferase sequence (pCMV-tau, pCMV-APP. pCMV- β-globin) were transfected into Daoy cells using lipofectamine 3000 as recommended by the manufacturer (Thermofisher). After 72 hours, plates were briefly washed in cold (4°C) PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate, 10mM potassium phosphate, pH 7.4) and then UV-crosslinked in a Stratalinker XL-1500 at 4000 mJ. Following crosslinking cells were harvested by scraping in cold PBS and collected by centrifuged at 200 x g for 5 minutes. Cells were lysed on ice for 15 minutes using mild lysis buffer (Imprint RIP kit, Sigma) that included RNAse inhibitor, protease inhibitor, and 0.1 mM dTT. Cell lysate was prepared by centrifugation at 16000 x g for 15 minutes at 4°C. A fraction (5%) of this supernatant was retained to serve as the input control. The remaining lysate was used for immunoprepicipations with mouse anti-PTB (Invitrogen), mouse anti-La (BD Biosciences), or rabbit anti-IgG (Cell signaling) antibodies added at a ratio of 10 µl of antibody to 180 µL lysate and 320 µL IP buffer (wash buffer with the addition of protease and RNAse inhibitors (included in the Imprint RIP kit)). Antibody binding was performed overnight at 4°C with continual rotation. Immunoprecipitation was performed by the addition of 20 µL of pre-washed protein G magnetic beads with additional rotation for 3 hours at 4°C. Complexes were recovered magnetically through the removal of supernatant followed by 4 washes with RIP Wash buffer (Imprint RIP kit). Total RNA isolation and RT-PCR were performed as described above to determine identity and specificity of protein:RNA interactions.

2.13 siRNA Human Kinase library screen

The Silencer® select human kinase library (Thermofisher) was used to target 710 human kinases. The library was to perform targeted knockdown in SKNSH cells, which were

subsequently screened for the impact of targeted knockdown on IRES-mediated translation using the monocistronic luciferase reporter system. Screening was performed based on manufacturer recommendations. Briefly, all experiments were performed using clear 96 well plates. The individual siRNA treatments were set up by adding 45 μl of serum free medium to each well followed by 2 μl of each stock siRNA mixed by pipetting up and down five times. Next, 3 μl of diluted INTERFER-in (0.5 μl INTERFER-in plus 2.5 μl of water per well) was added and mixed promptly by shaking the plate on a rotator for 5 mins. The plates were incubated for 15 minutes at room temperature before adding 6000 SKNSH cells, suspended in 150 μl of complete medium. Cells were then incubated at 37°C for 48 hours prior to RNA transfection. The medium was aspirated and 50 μl of serum free medium was added to each well. RNA transfection was performed as described (scaled down for 96-well plate) for 4 hours. The medium was then aspirated from the plates and 50 μL of 1X Passive lysis buffer (Promega) was added to each well. The plate was rocked at room temperature for 15 minutes, and 40 μl of the lysate were transferred to an opaque white 96-well plate for dual luciferase assay.

2.14 Statistical analysis

Data are presented as mean ± standard deviation. The number of repeats for each experiment (n) is specified in the corresponding figures. The p values were calculated by Student's t-test.

Chapter III

Regulation of tau IRES:

stress, kinases, and pathways

The tau mRNA is translated through both cap-dependent and cap-independent (IRES-dependent) mechanisms (Veo & Krushel, 2009). IRES-dependent translation is thought to be utilized when cap-dependent translation is inhibited. This occurs during normal physiological processes including mitosis, but also in response to stressful events such as apoptosis (Cornelis et al., 2000; Holcik & Sonenberg, 2005). Moreover, signaling pathways and molecules that regulate cap-dependent translation are also thought to be involved in IRES-dependent translation of some IRES containing mRNAs. In this chapter, I examine the regulation of tau IRES dependent translation during various cellular conditions, and attempt to identify regulatory networks involved in this regulation.

3.1 Development of assay

One of the first assays used to determine if a 5' leader consists an IRES is the dicistronic DNA construct assay (Macejak & Sarnow, 1991; Stoneley et al., 2000). In this assay, a DNA construct encoding a dicistronic message is transfected into cells and transcribed by an SV40 promoter (see Figure 2.1). The upstream cistron is translated in a cap dependent manner and serves as an internal control for transfection efficiency. The leader being tested for IRES activity is placed in the intercistronic region of the transcript, which will be translated only if an IRES is present. Many cellular IRESes were initially discovered using this assay(Komar & Hatzoglou, 2005). Unfortunately, many of these IRESes were determined to be a result of cryptic promoter activity, cryptic splicing, leaky scanning or reinitiation (Kozak, 2001). To circumvent this issue, many methods were devised to determine if the potential IRES activity was real (Van Eden, Byrd, Sherrill, & Lloyd, 2004). For example, northern blotting, or RT-PCR were used to compare the levels of RNA products generated from the two cistrons. Another method used siRNA targeting the

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upstream cistron to check whether translation of both cistrons is decreased by the same amount, which would indicate a single dicistronic construct was transcribed. Promoterless dicistronic constructs were also used to eliminate cryptic promoter activity but these constructs can often be unreliable, as translation of the upstream cistron was also sometimes detected.

Another approach more commonly used to identify IRESes is to transfect cells with *in vitro* transcribed dicistronic RNA constructs (Koev, Duncan, & Lai, 2002; Thompson & Sarnow, 2003). This method eliminates cryptic promoter and aberrant splicing activities. In addition, use of ApppG cap analog to replace the m7G cap eliminates the possibility of ribosomal shunting after recruitment by the cap structure or readthrough of the upstream stop codon. However, by inhibiting translation of the upstream cistron the use of an internal control for transfection efficiency is also lost.

The major limitation to using dicistronic RNA assays is that the majority of eukaryotic mRNA is monocistronic. Eukaryotic mRNAs are circular through protein interactions, which may promote translation near the cap in comparison to the IRES placed in the middle of the transcript. Further, the dicistronic construct might not be able to replicate potential interactions between the IRES and the 3'UTR. The most rigorous assay for identifying IRESes in a more physiological context is the monocistronic RNA assay. This assay utilizes transfection of a single *in vitro* transcribed ApppG capped and poly (A) tailed RNA message. In our assay we place the leader sequence upstream of the *Photinus* luciferase ORF as a reporter (Figure 2.2). A more detailed explanation of the assay is described below.

3.1A. Tau IRES is differentially utilized in different cells

One of the major factors contributing to differential regulation of internal translation

of many IRES containing mRNAs is the cellular environment such as ribosome availability, and the accessibility to other regulating factors. It was previously shown in our laboratory that the 5' UTR of tau mRNA contains an IRES, and that IRES-dependent translation contributes to the total level of translation in SKNSH (human neuroblastoma) cells (Veo & Krushel, 2009). Moving forward I wanted to first test if the tau IRES usage is dependent upon cellular context. First, I examined the tau IRES usage in various cell lines during normal condition. For this, I utilized the monocistronic *Photinus* luciferase construct containing the tau 5'UTR upstream of the *Photinus* ORF sequence. Plasmid was linearized downstream of a poly A stretch for *in vitro* transcription and capping with either an m7G cap or an ApppG cap (A-cap). The A-cap structure is not recognized by the cap-binding protein eIF4E, but still provides stability to the mRNA. In order to normalize the *Photinus* luciferase expression between transfections a m7G capped *Renilla* luciferase transcript sequence was used. Therefore in a standard assay m7G or A-capped RNA is cotransfected with m7G capped *Renilla* luciferase.

I first performed this assay using human SKNSH neuroblastoma cell line. The β-globin 5' UTR was used as a negative control while the APP, and EMCV IRESes were used as a positive control for the assay. Dual luciferase assay was performed and the ratio of *Photinus* luciferase to *Renilla* luciferase activity (P:R) was measured. In this assay, the P:R obtained using the m7G-capped mRNAs gives a measure of the total translation (including IRES dependent translation) by the given leader, while the A-capped mRNA gives a measure of the cap-independent (and thus IRES dependent) translation by the leader. The P:R ratio from the m7G capped RNA is set to 1, and the P:R ratio from the A-capped RNA is then normalized to this value to allow comparison between experiments and to establish the IRES

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usage for a given leader.

Using this method, it was shown that all of the translation by the β–globin leader is cap-dependent (since β–globin mRNA does not contain an IRES), while the IRES dependent translation by EMCV was comparable to its cap-dependent translation (since majority of the translation by the EMCV leader is mediated by its IRES) (Figure 3.1). The tau IRES utilization in comparison to m7g capped translation was 30%, while APP IRES utilization was 20% in these cells.
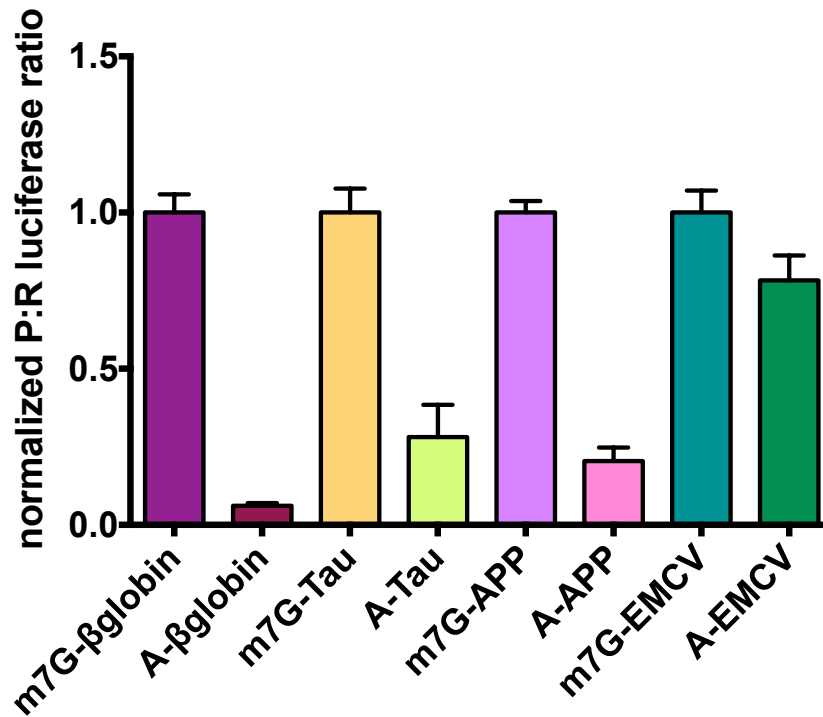
Figure 3.1. **Tau IRES utilization in SKNSH cells**. Monocistronic *Photinus* luciferase mRNA containing either tau, β-globin, APP, or EMCV 5' leader were capped with either an m7G or an ApppG cap and cotransfected with m7G capped *Renilla* luciferase into SKNSH cells. A ratio of *Photinus* luciferase activity to *Renilla* luciferase activity (P:R) was obtained for both ApppG capped and m7G capped mRNAs. The P:R ratio obtained from the m7G capped *Photinus* luciferase mRNA containing the various leaders and the m7G capped *Renilla* luciferase was set to 1 for each cell line. The P:R ratio obtained from the ApppG capped *Photinus* luciferase mRNA containing the various leaders and the m7G capped *Renilla* luciferase was normalized as a percentage of the m7G capped RNA (n=3). Error bars represent SD.

This assay was repeated in two other human neural cell lines (the U87 glioblastoma

cells, the Daoy medulloblastoma cells), one non-neural human cell line (the MCF12A breast

epithelial cells), and one neural rat cell line (the C6 glial cells). As expected, the EMCV

leader primarily translated through IRES dependent translation (Figure 3.2D), while the β–

globin leader translated through cap-dependent mechanism (Figure 3.2 B).
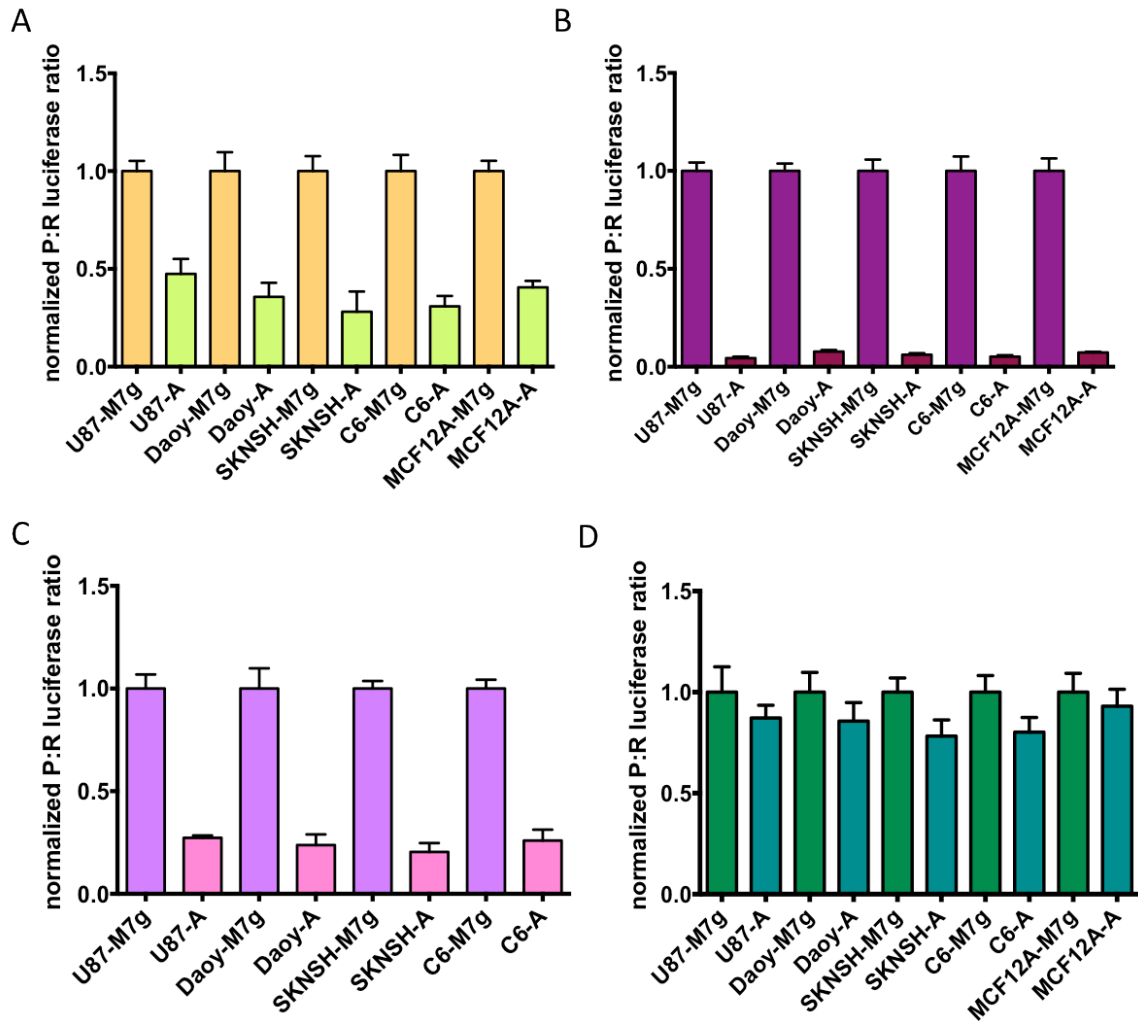
Figure 3.2. **Tau IRES is differentially utilized in various cell lines.** Monocistronic *Photinus* luciferase mRNA containing either tau (A), β-globin (B), APP (C), or EMCV (D) 5' leaders were capped with either an m7G or an ApppG cap and cotransfected with m7G capped *Renilla* luciferase into U87, Daoy, SKNSH, C6, or MCF12A cells. A ratio of *Photinus* luciferase activity to *Renilla* luciferase activity (P:R) was obtained for both ApppG capped and m7G capped mRNAs. The P:R ratio obtained from the m7G capped *Photinus* luciferase mRNA containing the various leaders and the m7G capped *Renilla* luciferase was set to 1 for each cell line. The P:R ratio obtained from the ApppG capped *Photinus* luciferase mRNA containing the various leaders and the m7G capped *Renilla* luciferase was normalized as a percentage of the m7G capped RNA (n=3). Error bars represent SD.

The tau IRES utilization varied in different cell lines, from 30% in SKNSH cell to almost 50% in the U87 cells, which had the highest tau IRES usage. Interestingly, tau IRES was utilized at a similar capacity in non-neuronal MCF12A cells as well as rat glial C6 cells (Figure 3.2 A). The APP IRES utilization was not tested in MCF12A cells, but the IRES usage was similar (~25-30%) in all the other cell lines tested (Figure 3.2 C). These results showed that tau IRES was utilized even in the presence of the m7g cap. In addition, comparison of the different cell lines showed that tau IRES is not differentially used among the different cell types including neural and non-neural cells. Daoy cells were used for the rest of the study (except for PTB and nPTB overexpression studies, where U87 cells were used) because tau IRES utilization is high in these cells. Daoy cells also have a faster doubling time, and are much efficient for RNA transfection compared to other cell lines.

3.2 Role of cellular stressors on Tau IRES dependent translation

IRES-dependent translation of many viral and cellular mRNAs is upregulated in response to cellular stress (Nevins et al., 2003). Stress conditions that have been shown to regulate the IRES dependent translation include: hypoxia, nutrient deprivation, apoptosis, oxidative stress, UV-irradiation, and viral infection. This upregulation is thought in part to be a response to the global inhibition of cap-dependent protein synthesis mediated through pathways that inhibit mTOR and PERK kinases, and their downstream effector proteins 4EBP and elF2α respectively (Donnelly et al., 2013; Richter & Sonenberg, 2005; Sonenberg & Hinnebusch, 2009). The high utilization of oxygen and the absence of a strong antioxidant defense mechanism in the brain makes it uniquely prone to oxidative stress. For this reason IRES-mediated translation is thought to play a protective role in neurons. The fact that persistent oxidative stress is seen in a number of neurodegenerative disease has led to the

hypothesis that it may be one of the key early events in disease pathogenesis (Butterfield, Perluigi, & Sultana, 2006; Naini & Soussi-Yanicostas, 2015). In many cases, oxidative stress is exacerbated by the accumulation of redox metals including iron (X. Chen, Guo, & Kong, 2012; Uttara, Singh, Zamboni, & Mahajan, 2009). In addition, there is often evidence of acute ischemic injury in the brains of ND patients. Under ischemic conditions, there is oxidative stress due to an increase in the production of free radicals, which leads to endoplasmic reticulum stress and the activation of the unfolded protein response (UPR) (DeGracia & Montie, 2004; Nita et al., 2001). UPR causes PERK mediated phosphorylation of eiF2α, which reduces cap-dependent translation and increases IRES dependent translation of select mRNAs.

In AD, the Aβ cascade hypothesis postulates that activation of stress response kinases such as JNK1 by Aβ initiates the process of tau pathology (Karran, Mercken, & De Strooper, 2011; Spillantini & Goedert, 2013). Studies have shown that the addition of exogenous Aβ peptide to cultured cells increases tau phosphorylation (Hernandez, Gomez de Barreda, Fuster-Matanzo, Lucas, & Avila, 2010).

Previous studies done by our lab showed that chemically induced ischemia, and increase in extracellular iron levels lead to an increase in APP protein levels and IRES activity (Beaudoin et al., 2008). Taken together, these studies led us to question whether the tau IRES is regulated in response to cellular stress conditions as seen with other IRESes. More importantly, I wanted to test whether stress conditions that mimic the ones commonly observed in NDs such as iron induced oxidative stress, ischemia, viral-infection, and the presence of extracellular Aβ peptides would increase IRES dependent translation of tau, and alter tau protein expression.

3.2A Tau IRES activity is unaffected by chemically induced ischemic conditions

In order to investigate whether tau IRES is regulated by ischemia, a combination of various chemicals was used to mimic ischemic conditions in SKNSH cells. Sodium azide, which inhibits oxidative phosphorylation, was used to induce chemical anoxia, in combination with 2-deoxyglucose, which inhibits glycolysis (Imura et al., 1999). Cells were treated with chemical ischemia reagents for 10 minutes, and tau IRES activity was examined using the reporter assay developed in section 1. In cells treated with chemical ischemia reagents, tau IRES activity remained at similar level as untreated cells. As a positive control, APP leader was used. APP IRES dependent translation was previously shown to be upregulated by the addition of ischemic reagents. Indeed, chemical ischemia led to a significant 1.4 fold increase in APP IRES activity ($p<0.005$), which was similar to previously published results (Beaudoin et al., 2008). These results suggest that this chemically induced condition is not sufficient to elicit an increase in tau IRES activity. It is possible that addition of other stress conditions in combination with ischemia, as observed in pathologic conditions, is required to increase tau IRES dependent translation. Alternatively, tau IRES activity may already be maximally stimulated given that untreated and treated ratios are similar to those observed for treated APP IRES use.
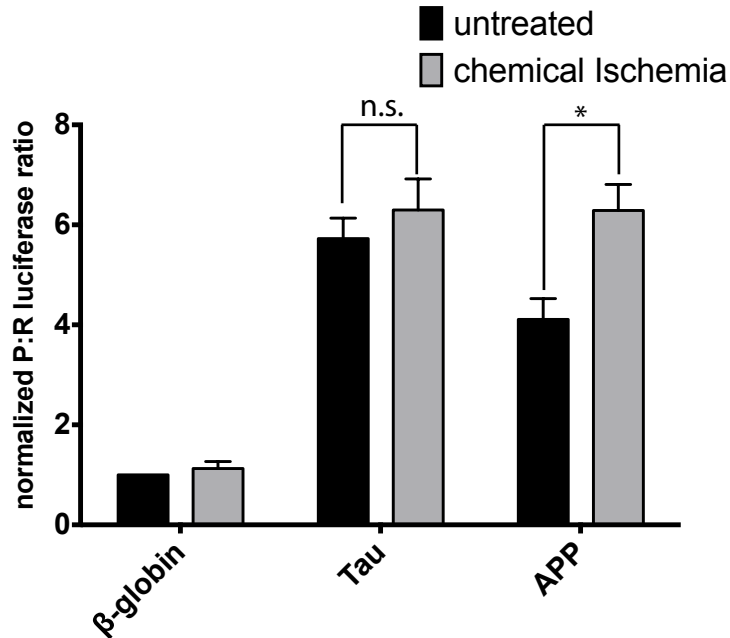
Figure 3.3. **Chemical ischemia does not affect tau IRES activity.** SKNSH cells were either untreated or treated with an acute 10 min exposure to 10 mM sodium azide and 10mM 2-deoxyglucose. Monocistronic luciferase RNAs containing the β-globin, tau, or APP 5' leaders upstream of the *Photinus* luciferase gene were *in vitro* transcribed, capped with an ApppG, and tailed. All RNAs were co-transfected into the cells with an m7G capped *Renilla* luciferase mRNA as transfection control. After 4 hours, dual luciferase assay was performed. The *Photinus*: *Renilla* luciferase ratios (P:R) are normalized to the activity obtained from the mRNA containing the β -globin 5' leader (n=3). Error bars represent SD. * $p < 0.005$.

3.2B Tau IRES is enhanced by extracellular iron

Next, I examined whether increased iron affects the tau IRES. Cells were treated with ferric ammonium citrate (FAC). FAC is a soluble ferric iron complex, which has been shown to increase the level of intracellular iron in neural cells (W. R. Martin, Ye, & Allen, 1998). Following treatment, cells were transfected as previously described. Treatment with FAC increased tau IRES activity by 2 fold (Figure 3.4 A). APP IRES activity was increased by ~2 fold with FAC treatment, as previously described (Beaudoin et al., 2008). It was previously shown that along with increasing APP IRES dependent translation, increased iron levels also elevated endogenous APP protein expression ((Beaudoin et al., 2008), also see Figure 3.4 B,

C). Analysis of endogenous tau protein levels by western blot showed that tau expression remained unchanged with increased iron by FAC treatment.
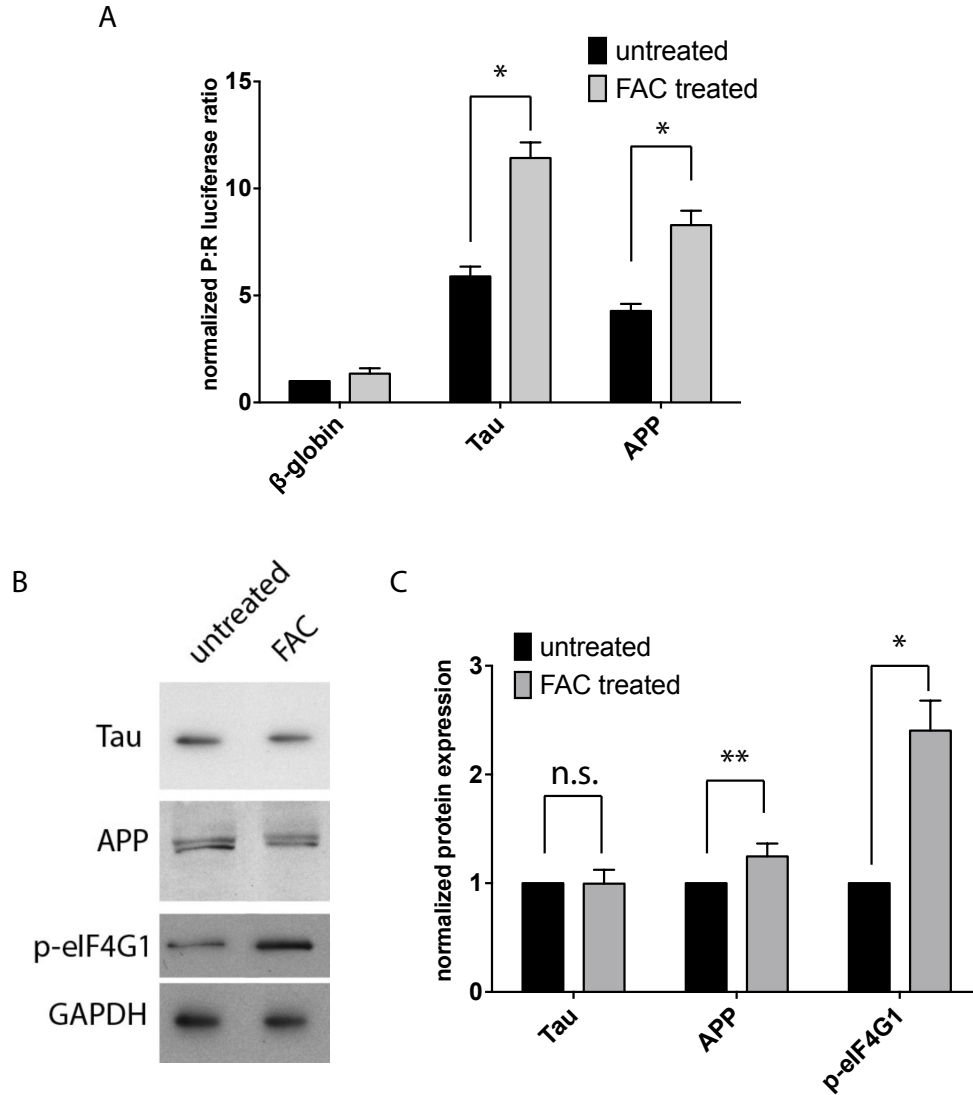
Figure 3.4. **Iron exposure increases tau 5' leader IRES activity.** SKNSH cells were treated with either DMSO or ferric ammonium citrate (FAC) supplemented media for 24 hours. (A) Monocistronic *Photinus* luciferase A-capped RNAs containing the β-globin, tau, or APP 5' leaders were co-transfected into the cells with an m7G capped *Renilla* luciferase mRNA. After 4 hours, dual luciferase assay was performed. The *Photinus*: *Renilla* luciferase ratios (P:R) (+S.D.) are normalized to the activity obtained from the mRNA containing the β -globin 5' leader (n=3). (B) Lysates from DMSO treated or FAC treated cells were examined for Tau, APP, p-eIF4G1, and GAPDH proteins using western blotting. (C) The level of Tau, APP and p-eIF41G1 was quantitated and normalized to GAPDH levels. Error bars represent SD. * $p < 0.0008$, ** $p < 0.03$.

As a marker for inhibition of cap-dependent translation, phosphorylation of eIF4G1 was observed. EIF4G1 is phosphorylated by different kinases, including Pak2 during conditions of stress thereby inhibiting cap-dependent translation (Ling, Morley, & Traugh, 2005). As expected, phosphorylation of eIF4G1 was increased following treatment with FAC. These results suggest that iron stimulates cap-independent translation by tau IRES. Since there was no change observed in endogenous tau protein levels during these conditions, it suggests that maintaining tau protein levels is essential, and that in the absence of cap-dependent translation, IRES-dependent translation takes over to maintain the levels of tau protein.

3.2C Tau IRES is enhanced in response to increased poly (I:C) treatment

Next I examined whether double stranded RNA (dsRNA) affects tau IRES mediated translation. Again, SKNSH cells were utilized. Cells Ire treated with 500 μg/ml polyinosinic : polycytidylic acid (poly (I:C)). Poly (I:C) is a double stranded RNA analog, and mimics dsRNA virus infection by activating PKR kinase pathway to phosphorylate eIF2α and inhibit cap-dependent translation. Following treatment, cells were transfected (as previously described) using *Photinus* luciferase RNA with β-globin, tau, or APP 5' leaders. Poly (I:C) treatment increased tau IRES dependent translation by ~ 1.2 fold respectively. Poly (I:C) treatment increased APP IRES activity by 1.2 fold, which was similar to the levels observed for tau IRES. Examination of tau protein expression in poly (I:C) treated lysates showed that tau protein expression did not change despite the increase in IRES activity, while an increase in APP protein expression was observed (Figure 3.5 B, C). These results further imply that perhaps during conditions when cap-dependent translation is reduced such as during dsRNA viral infection, tau IRES is utilized to maintain the tau protein levels.
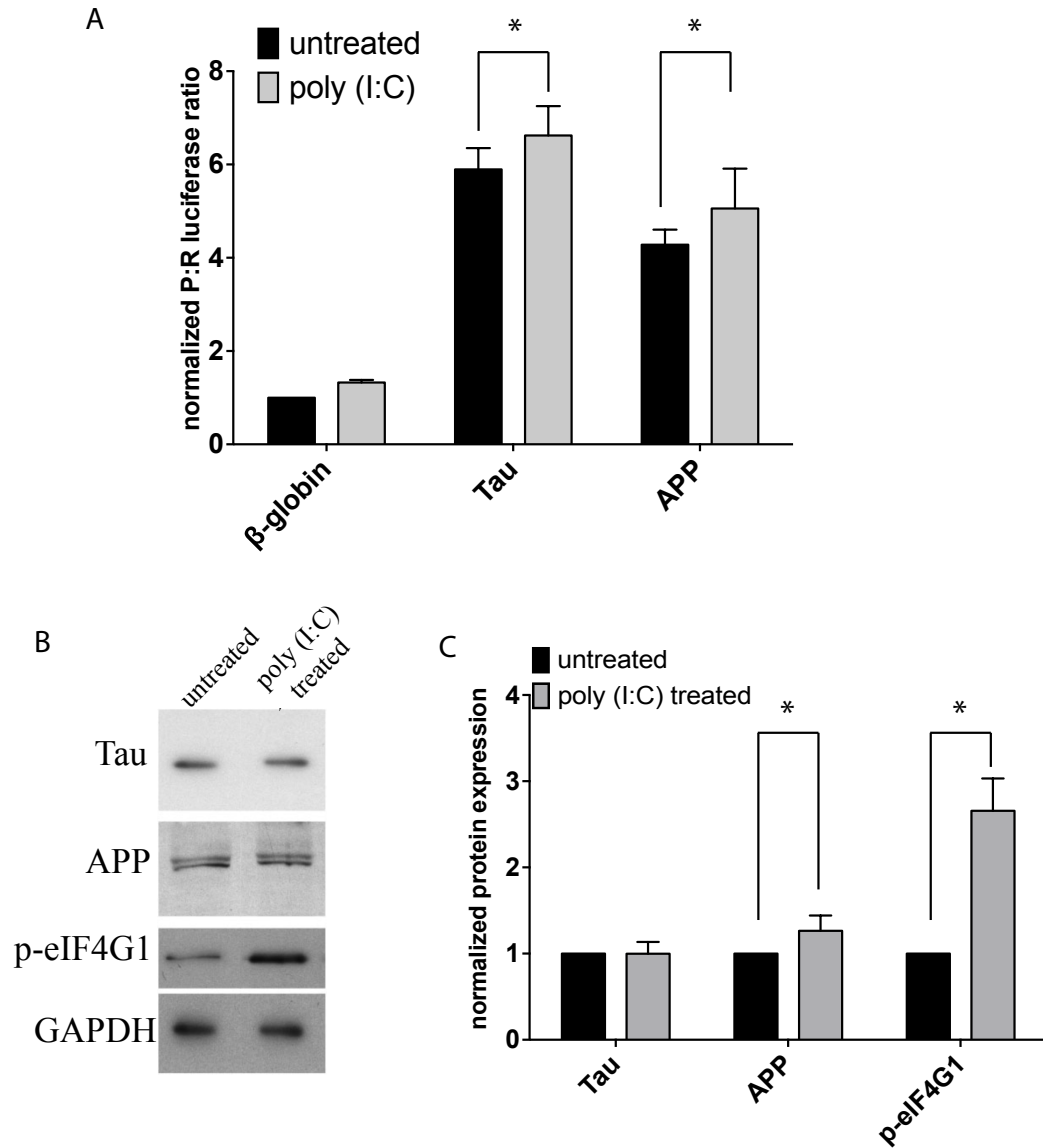
Figure 3.5. **Poly (I:C) treatment increases tau 5' leader IRES activity.** SKNSH cells were treated with either DMSO, or 500 ug/mL of poly (I:C). (A) Monocistronic luciferase RNAs containing the β-globin, tau, or APP 5' leaders upstream of the *Photinus* luciferase gene were *in vitro* transcribed, capped with an ApppG, and tailed. All RNAs were co-transfected into the cells with an m7G capped *Renilla* luciferase mRNA as transfection control. After 4 hours, dual luciferase assay was performed. The *Photinus*: *Renilla* luciferase ratios (P:R) are normalized to the activity obtained from the mRNA containing the β -globin 5' leader (n=3). (B) Lysates from DMSO treated or poly (I:C) treated cells were examined for Tau, APP, p-eIF4G1, and GAPDH proteins using western blotting. (C) The level of Tau, APP and p-eIF41G1 was quantitated and normalized to GAPDH levels. Error bars represent SD. * p < 0.05

3.2D Aβ (1-42) peptide increases tau IRES dependent translation

I wanted to test whether addition of extracellular Aβ peptides also increases tau IRES

dependent translation. Previous studies have shown that addition of Aβ peptide to cultured

cells increases phosphorylation levels of tau as well as total tau protein levels (Hernandez et

al., 2010; Moore et al., 2015). For this study, SKNSH cells were either left untreated or

treated with Aβ (1-42) peptide overnight. Cells were then transfected as previously described

using luciferase mRNA with β-globin, tau, or APP leaders. The P:R ratio obtained from this

assay showed that presence of Aβ (1-42) increased IRES dependent translation of both tau

and APP by 1.4 fold and 1.7 fold respectively (Figure 3.6).
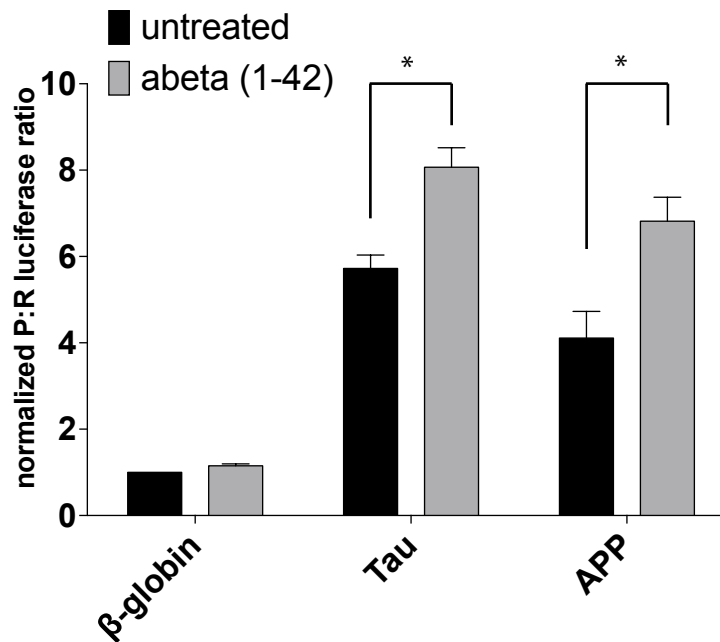
Figure 3.6. **Extracellular Aβ peptide increases tau 5' leader IRES activity.** SKNSH cells
were treated with either DMSO or Aβ (1-42) peptide overnight. Monocistronic luciferase
RNAs containing the β-globin, tau, or APP 5' leaders upstream of the *Photinus* luciferase
gene were *in vitro* transcribed, capped with an ApppG, and tailed. All RNAs were co-
transfected into the cells with an m7G capped *Renilla* luciferase mRNA as transfection
control. After 4 hours, dual luciferase assay was performed The *Photinus*: *Renilla* luciferase
ratios (P:R) are normalized to the activity obtained from the mRNA containing the β -globin
5' leader (n=3). Error bars represent SD. * p < 0.005.

Taken together with our previous knowledge about the role of Aβ peptides in regulating tau protein expression and phosphorylation, these results suggest that elevated IRES dependent translation of tau is one of the mechanisms by which tau protein level is increased in the presence of extracellular Aβ peptides. These results implicate tau IRES mediated translation as one of the mechanisms leading to tau pathogenicity.

3.3 Examining cellular signaling pathways for mediators of IRES-dependent translation

Cap dependent translation is influenced by the cellular environment. Upstream mitogenic factors including growth factors, and cytokines converge on translation through various signaling pathways such as the PI3/Akt/mTOR and the MAPK signaling pathways. Growth factors and insulin stimulation activate the PI3/AKT/mTOR pathway, which causes a global up-regulation of cap-dependent translation via the phosphorylation of 4EBP1 and S6K (X. M. Ma & Blenis, 2009; Thoreen et al., 2012). On the other hand, the MAPK signaling also enhances mTOR mediated cap-dependent translation through activation of ERK and RSK (Goetz, Everson, Zhang, & Gromeier, 2010). In addition to regulating translation through the mTOR pathway, many of the upstream kinases also phosphorylate various eukaryotic initiation factors, providing additional regulation of cap-dependent translation. For example, activation of MAPK leads to phosphorylation of eIF4B by RSK (Galan et al., 2014). Phosphorylated eIF4B is implicated in facilitating translation by acting as a cofactor for eIF4A RNA helicase. This phosphorylation of eIF4B also promotes association between eIF4A and eIF3, which further promotes translation (Galan et al., 2014; G. W. Rogers, Jr. et al., 2001; Shahbazian et al., 2006).

Many of the growth factors and signaling molecules that influence cap-dependent translation have also been shown to modulate IRES mediated translation of some mRNAs.

The activity of cyclin D1, and c-myc IRESes are upregulated by the activation of AKT signaling (Shi et al., 2005). Moreover, inhibiting the mTORC1 pathway by rapamycin further enhances these IRESes in a p38MAPK and ERK dependent manner (Shi et al., 2005). Despite these studies, no general signaling pathway regulating IRES-dependent translation has been elucidated.

The goal of this and the subsequent sections was to identify potential regulators of the tau IRES. This section focuses on growth factor pathways as triggers of IRES dependent translation of tau.

3.3A Tau IRES activity is not increased in response to growth factors

Previous studies done in our lab have shown that the AURKA IRES is differentially influenced by growth factors such as EGF, and bFGF via the MAPK pathway (Dobson et al., 2013). I wanted to examine whether the tau IRES is also regulated by the action of these signaling molecules. The medulloblastoma Daoy cell line, which has tau IRES activity (figure 3.1), were utilized because they are known to express receptors to all the factors used in this study. Tau IRES activity was examined after addition of EGF, IGF-1 insulin, bFGF, and BDNF. The P:R luciferase ratio for tau IRES was reduced for growth factor treated cells compared to untreated cells, with the greatest reduction seen in EGF, bFGF, and BDNF treated cells, as shown in Figure 3.7 A. Western blotting was performed to detect phosphorylation of eFI4E, which showed that this phosphorylation was increased in cells treated with EGF, bFGF, and BDNF (Figure 3.7 B,C). This data correlated with the reduced P:R ratio in the corresponding cells.
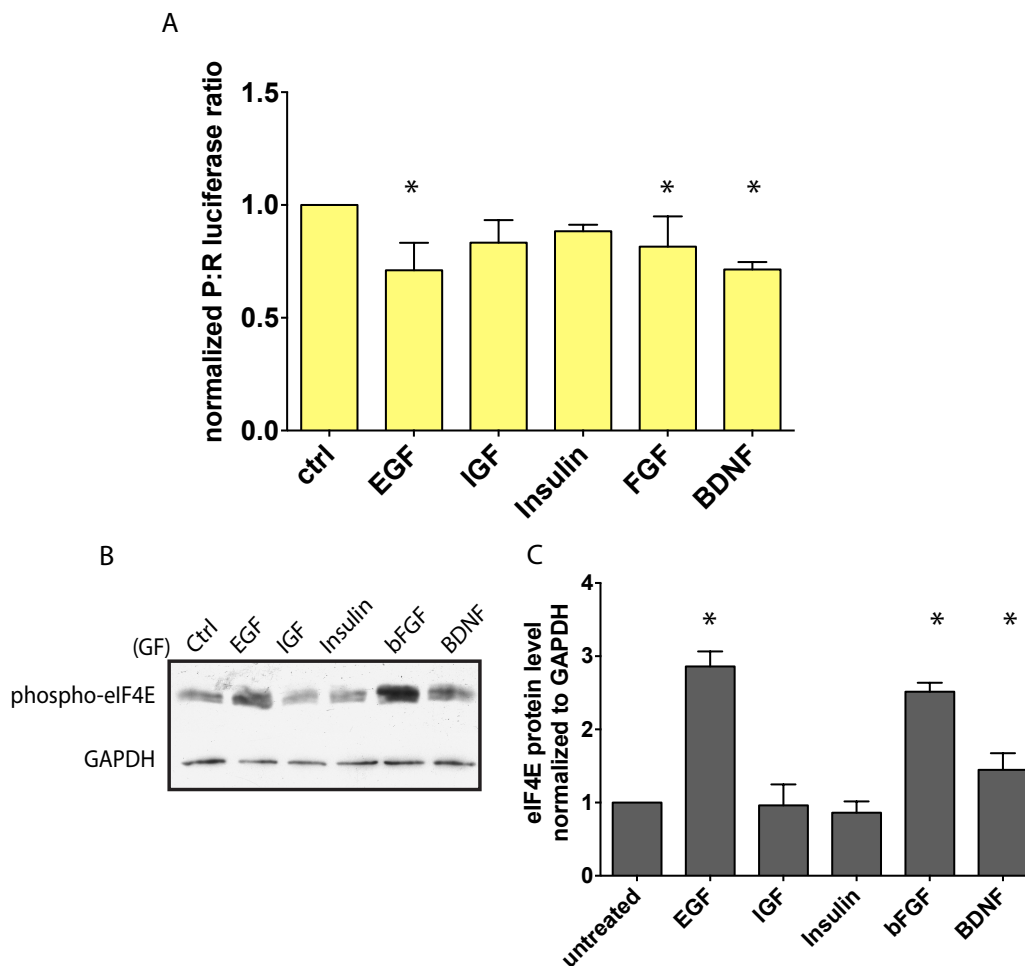
Figure 3.7. **Tau IRES activity does not increase in response to growth factors**. Daoy cells were serum starved for four hours and either left untreated or treated with EGF, IGF-1, Insulin, bFGF, or BDNF as described. A) After three hours, cells were transfected with ApppG capped monocistronic *Photinus* luciferase mRNAs containing the tau 5' leader along with m7G capped *Renilla* luciferase mRNA. Dual luciferase assay was performed and the ratio of the *Photinus*: *Renilla* luciferase activity (P:R) (n=3)were normalized to the untreated cells. Lysate was obtained from untreated, or growth factor treated cells, and western blotting was performed to detect the phosphorylation of eIF4E (B), which was quantitated and normalized to GAPDH levels (C). Error bars represent SD. * $p < 0.05$.

Following these experiments, a major question arose during the interpretation of the

data. While the P:R ratio is a good measure for comparing IRES activity in some assays, this

method only works under the assumption that the m7g cap dependent translation (measured

as activity of *Renilla* luciferase) is not affected in the experimental conditions. However, in

conditions where cap-dependent translation is altered, such as with growth factor treatment,

the P:R ratio may not accurately represent the level of IRES activity. Addition of growth factors increases cap-dependent translation (and thus the *Renilla* luciferase activity) via the PI3K-mTOR pathway or the MAPK pathways. So the reduced P:R ratio observed with the addition of growth factors (Figure 3.7 A) could be due to enhanced *Renilla* luciferase activity.

To address this potential problem, I reanalyzed the dataset examining either the *Photinus* luciferase activity or the *Renilla* luciferase activity individually, and compared them to the P:R ratio. As predicted, *Renilla* luciferase activity, which is a measure of cap-dependent translation, was increased following treatment with EGF, IGF-1, and BDNF by 13%, 23%, and 31% respectively, compared to untreated cells ($p<0.05$) (Figure 3.8 A, blue bars). Addition of insulin, and bFGF did not increase *Renilla* luciferase activity.

Interestingly, despite an increase in *Renilla* luciferase activity, addition of EGF, and IGF1 did not increase tau IRES activity (Figure 3.8 A, red bars), indicating that tau IRES activity was not affected by these growth factors. In fact, addition of bFGF led to a decrease in *Photinus* luciferase activity (37%), while addition of BDNF did not alter *Photinus* luciferase activity even though *Renilla* luciferase activity was increased. BDNF treatment is a good example of why P:R ratio does not provide a good measure IRES dependent translation in conditions that also alter cap-dependent translation. Here the reduction in the P:R ratio is clearly due to increased cap dependent translation and not due to a reduced IRES dependent translation. I also examined the regulation of APP IRES dependent translation by these growth factors. As expected, addition of EGF and bFGF reduced activity of *Photinus* luciferase with APP leader compared to untreated cells by 18% and 14% respectively (Figure 3.8 B, red bars).
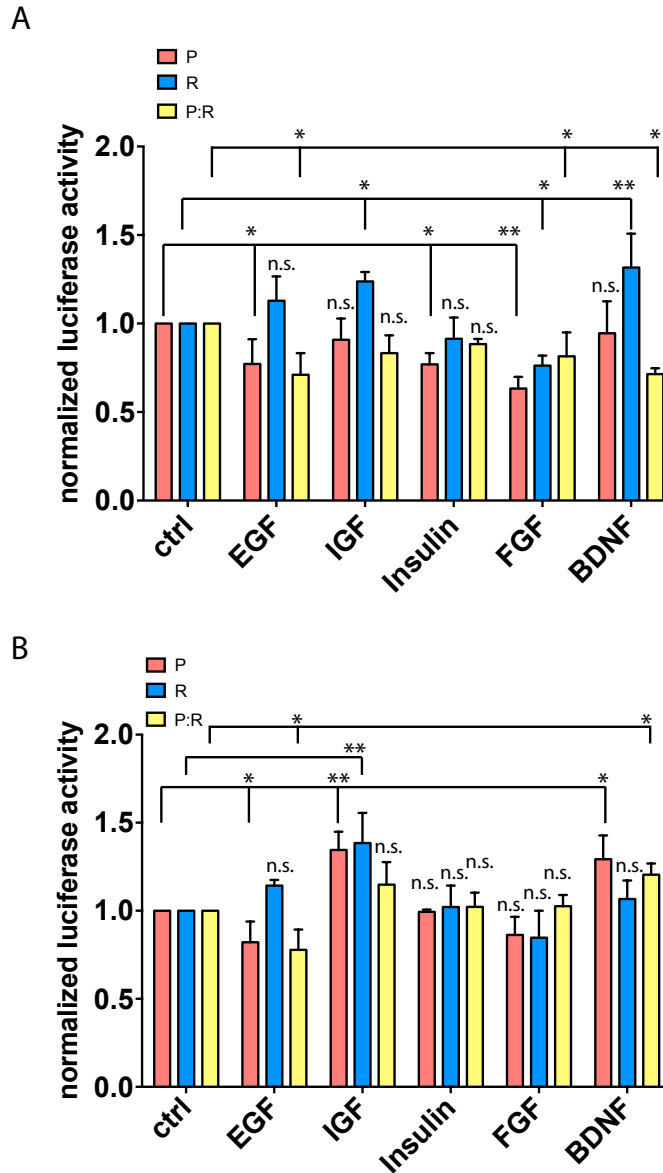
Figure 3.8. **Comparison of measurement of tau IRES**. Daoy cells were serum starved for four hours and either left untreated or treated with EGF, IGF-1, Insulin, bFGF, or BDNF as described. After three hours, cells were transfected with ApppG capped monocistronic *Photinus* luciferase mRNAs containing the either the tau 5' leader (A), or the APP 5' leader (B) along with m7G capped *Renilla* luciferase mRNA. A comparison of the *Photinus* luciferase (P) (red bars), the *Renilla* luciferase (R) (blue bars), or a ratio of P:R (yellow bars) was done for each leader by setting the value obtained from the untreated cells to 1. The luciferase activity for the growth factor treated cells were normalized to the untreated cells. (n=3). Error bars represent SD. * $p < 0.05$, ** $p < 0.002$.

Addition of IGF-1, and BDNF, however, increased *Photinus* luciferase activity by

about 30% ($p < 0.05$). Since APP protein has a reported half-life of about 30 minutes

(Beaudoin et al., 2008), I examined whether endogenous APP protein expression was altered by treatment with these growth factors. Western blotting using APP antibody showed that the endogenous APP expression was increased following treatment with all of the growth factors used (p<0.05) (Figure 3.9 A, B). This increase is likely due to enhanced cap-dependent translation by the growth factors, although the increased IRES dependent translation by addition of IGF-1 and BDNF could also have contributed to APP expression.
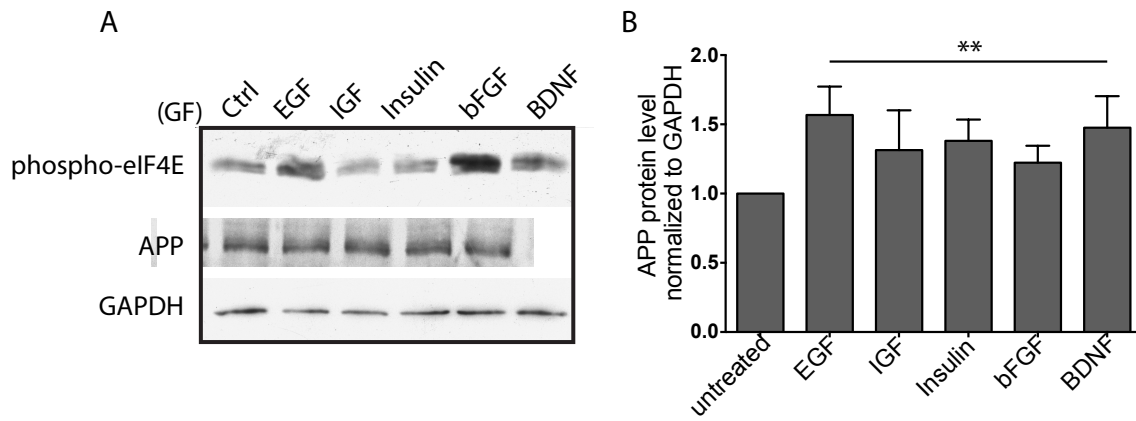
Figure 3.9. **Endogenous APP protein level is increased after growth factor addition**. Daoy cells were serum starved for four hours and either left untreated or treated with EGF, IGF-1, Insulin, bFGF, or BDNF as described. Lysate obtained from untreated, or growth factor treated cells, were analyzed for expression of APP, and phosphorylation of eIF4E (A). The level of APP protein was quantitated and normalized to GAPDH levels (B). Error bars represent SD. ** $p < 0.005$.

Since tau protein expression is most abundant in neurons, neuronal specific factors could affect tau IRES dependent translation. For this reason I examined IRES activity in response to treatment with neurotrophic factors glial cell line derived neurotrophic factor (GDNF), neurturin, heregulin β1 (Hrg-1β), and artemin. Similar to the examination of growth factors, I examined the effect of treating transfected Daoy cells with these neurotrophs.
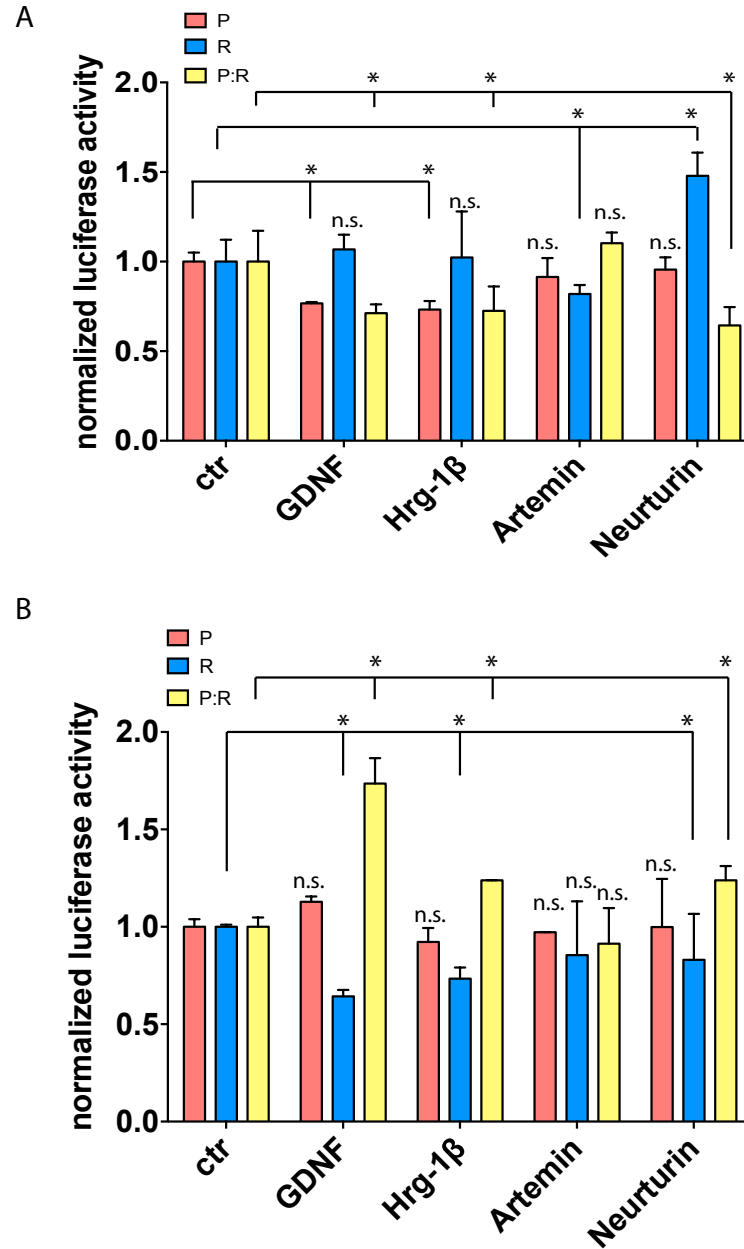
Figure 3.10. **Tau IRES activity is not increased by neuronal factors**. Daoy cells were either untreated or treated with GDNF, Hrg-1β, artemin, or neurturin as described. After three hours, cells were transfected with ApppG capped monocistronic *Photinus* luciferase mRNAs containing the either the tau 5' leader (A), or the APP 5' leader (B) along with m7G capped *Renilla* luciferase mRNA. A comparison of the *Photinus* luciferase (P, red bars), the *Renilla* luciferase (R, blue bars), or a ratio of P:R (yellow bars) was done for each leader by setting the value obtained from the untreated cells to 1. The luciferase activity for the growth factor treated cells was normalized to the untreated cells. (n=3). Error bars represent SD. * p < 0.005.

Interestingly, treatment with these factors did not cause an increase in *Renilla* luciferase activity (Figure 3.10 A, blue bars). Addition of GDNF, and Hrg-1β reduced *Photinus* luciferase activity of tau leader containing mRNA by 24, and 27% respectively (Figure 3.10 A, red bars), while artemin, and neurturin did not alter *Photinus* luciferase activity. Addition of these factors did not affect *Photinus* luciferase activity of APP leader containing mRNA (Figure 3.10 B, red bars).

These results clearly show that tau IRES dependent translation is differentially affected by various growth factors. In general, addition of growth factors reduced IRES dependent translation. This is likely due to an increase in cap-dependent translation during these conditions, which would potentially limit ribosome and other initiation factors to be utilized for IRES dependent translation. These results also show that the regulation of IRES mediated translation is not uniform across all IRESes, as both tau and APP IRESes were differentially regulated by different factors, suggesting that additional level of regulation exists in controlling individual IRESes.

3.3B Examining whether signaling pathways regulate tau IRES mediated translation.

Upstream stimuli regulate cap dependent translation through the action of various signaling kinases including those in the PI3K-Akt and the MAPK pathways. In order to examine the role of these signaling cascades in tau IRES mediated translation, I utilized pharmacologic inhibitors to block different kinases in these pathways. Daoy cells were treated with inhibitors to c-Jun N-terminal kinase (JNK), p70 ribosomal protein S6 kinase 1 (S6K), p38 MAPK, p90 ribosomal protein S6 kinase (RSK), PI3K, Mitogen-activated protein kinase kinase (MEK) 1 and 2, and MAPK interacting protein kinases (MNK) 1 and 2, and Akt kinases. In addition, inhibitors to EGFR and GSK3β were also used (Refer to Table 2.2,

and figure 2.2). Individual *Photinus* and *Renilla* luciferase activity were examined for the inhibitor treated cells, along with P:R ratio. In addition, a ratio of the *Photinus* luciferase activity to the amount of *Photinus* RNA (P:RNA) was also examined. In conditions where cap-dependent translation is altered, P:RNA might be a better indicator of IRES activity, as it gives a measure of the total translation of a reporter protein from a given amount of RNA.

Similar to the addition of growth factors blocking of their downstream pathways is also predicted to impact cap-dependent translation. Inhibition of JNK, S6K, p38 MAPK, RSK, PI3K, Akt, and GSK3β kinases reduced both cap-dependent and IRES-dependent translation by the tau IRES as shown by the reduced *Photinus* and *Renilla* luciferase activity (Figure 3.11 A, 3.12 A, blue bars, red bars). Inhibition of MNK 1 and 2 kinases, MEK 1 and 2 kinases, or EGFR did not affect cap-dependent, or IRES-dependent translation by tau leader. In an attempt to obtain a more precise measure of IRES dependent translation I examined the P:RNA ratio. This value was actually found to be comparable to the individual *Photinus* luciferase activity for most of the inhibitors (Figure 3.11, 3.12 pink bars). Clearly, P:R ratio was not able to depict the results from these experiments (Figure 3.11, 3.12, yellow bars). For tau IRES dependent translation, the biggest reduction was seen with RSK, and Akt inhibitors, which decreased the IRES activity by more than 90% compared to untreated cells. However, a closer examination of the raw RNA numbers showed that these drugs not only affected translation, but also affected RNA stability, which further reduced translation. Most of these inhibitors affected APP IRES dependent translation in a manner similar to the regulation of tau IRES. One outlier was the S6K inhibitor, which decreased cap-dependent translation by 40%, but increased APP IRES dependent translation (P: RNA) by ~25%.
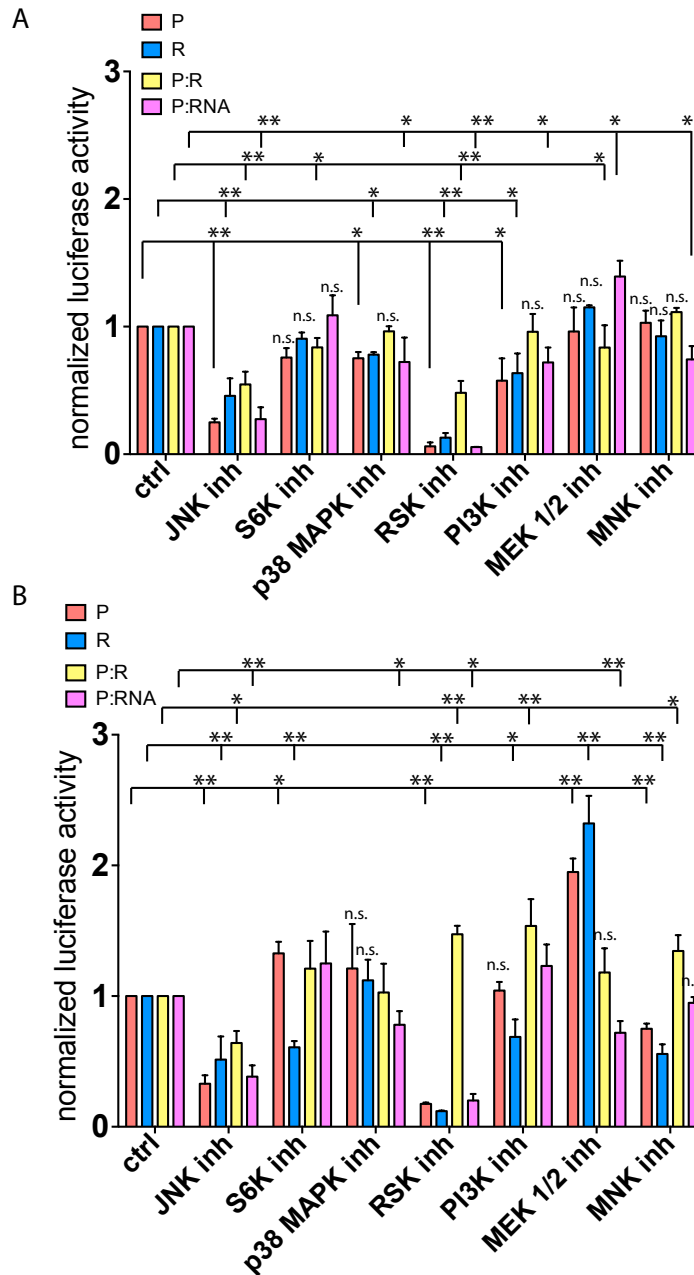
Figure 3.11. **Tau IRES activity is reduced by pharmacologic inhibition of various kinases in the PI3K and MAPK signaling pathways**. Daoy cells were either untreated or treated with inhibitors to JNK, S6K, p38 MAPK, RSK, PI3K, MEK1/2 and MNK 1/2 kinases. After three hours, cells were transfected with ApppG capped *Photinus* luciferase mRNAs containing either the tau (A) or the APP (B) 5' leader along with m7G capped *Renilla* luciferase mRNA. A comparison of the *Photinus* luciferase (P, red bars), the *Renilla* luciferase (R, blue bars), a ratio of P:R (yellow bars), and a ratio of *Photinus* luciferase activity to RNA level (P:RNA, pink bars) was done for each leader by setting the value obtained from the untreated cells to 1. The luciferase activity for the inhibitor treated cells was normalized to the untreated cells. (n=3). Error bars represent SD. * p < 0.01, ** p<0.005.
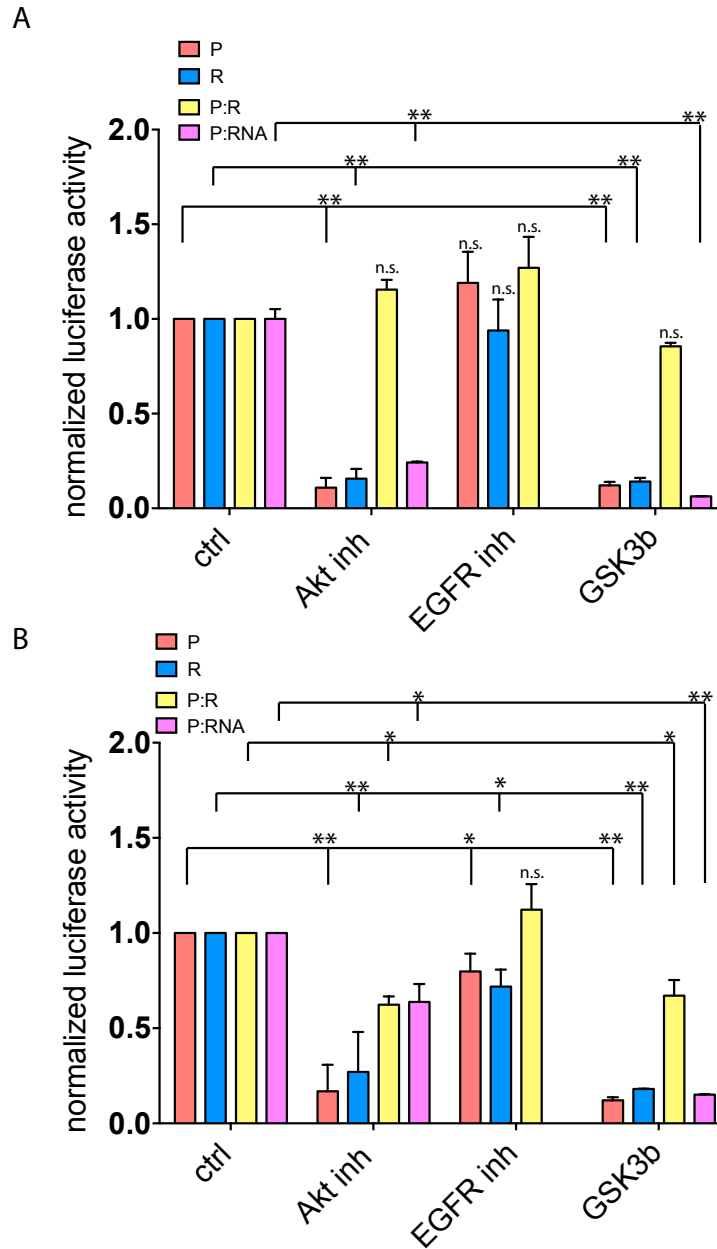
Figure 3.12. **Tau IRES activity is reduced by pharmacologic inhibition of various kinases and EGFR.** Daoy cells were either untreated or treated with inhibitors to Akt, EGFR, and GSK3β. After three hours, cells were transfected with ApppG capped monocistronic *Photinus* luciferase mRNAs containing the either the tau 5' leader (A), or the APP 5' leader (B) along with m7G capped *Renilla* luciferase mRNA. A comparison of the *Photinus* luciferase (P, red bars), the *Renilla* luciferase (R, blue bars), a ratio of P:R (yellow bars), and a ratio of *Photinus* luciferase activity to *Photinus* RNA level (P:RNA, pink bars) was done for each leader by setting the value obtained from the untreated cells to 1. The luciferase activity for the inhibitors treated cells were normalized to the untreated cells. (n=3). Error bars represent SD. * $p < 0.005$, *$p < 0.0001$.

These data show that the signaling pathways downstream of growth factor pathways modulate both the cap-dependent and IRES-dependent translation suggesting that these two translation mechanisms share many common upstream regulatory factors. Moreover, these results suggest that specific regulation of IRES dependent translation likely occurs downstream of these pathways. A more detailed analysis of these pathways and their downstream effectors is required to dissect the regulatory network for tau IRES mediated translation. Alternatively, it is possible that these pathways only affect cap-dependent translation, and the effect on the IRES is a secondary effect as a result of reduced cap-dependent translation.

3.4 Role of mTOR signaling in mediating translation by the tau IRES

Regulation of cap-dependent translation by the mTOR pathway has been well established. The mTOR pathway responds to cues from upstream signaling molecules and coordinates cell growth, proliferation, and metabolism by acting on protein synthesis (Inoki et al., 2012; X. M. Ma & Blenis, 2009). The mTOR protein exists in two functional complexes- mTORC1 and mTORC2. The mTORC1 has been shown to regulate protein synthesis via multiple downstream effectors, while mTORC2 is associated with cellular metabolism, cytoskeletal organization, and cell survival, but its role in regulation of protein synthesis is not well understood (Shimobayashi & Hall, 2014; Thoreen et al., 2012; Zoncu et al., 2011). Activation of mTORC1 controls protein synthesis via two major downstream effectors - the S6 kinases (S6Ks), and eIF4E-binding proteins (4E-BPs) (Kaizuka et al., 2010; Laplante & Sabatini, 2012; Zoncu et al., 2011).

Since both cap-dependent, and IRES-dependent translation pathways share many of the same upstream regulators as well as downstream initiation factors, mTOR signaling has

been implicated in regulating IRES-dependent translation as well. For example, inhibition of mTOR by its allosteric inhibitor rapamycin has been shown to enhance IRES dependent translation of cyclin D1 and c-myc (Shi et al., 2005). The goal of this section was to investigate the role of mTOR signaling in tau IRES dependent translation.

3.4A Role of Raptor and Rictor proteins

I first investigated the role of mTOR signaling by knocking down two important proteins– the regulatory-associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (Rictor). Raptor and Rictor are proteins specific to the two mTOR complexes- mTORC1 and mTORC2. siRNA mediated knockdown in Daoy cells led to a reduction of Raptor and Rictor protein by 60 and 40% respectively (Figure 3.13 A). Knocking down Raptor led to a 57% decrease in endogenous tau protein level and a 40% decrease in APP protein level, while reduction of Rictor led to a 15% decrease in tau protein level and 30% decrease in APP protein level (Figure 3.13 B).

I then used the reporter assay to examine if knockdown of these proteins affected tau IRES dependent translation. Knockdown of Raptor led to about 50% reduction in tau IRES dependent translation, as shown in Figure 3.13 C. Interestingly, APP IRES dependent translation was not affected by Raptor knockdown. Rictor knockdown did not affect either tau or APP IRES dependent translation. These results suggest the involvement of the mTORC1 complex in mediating protein synthesis by the tau IRES. It is possible that IRES dependent translation requires many of the same factors downstream of mTORC1 complex as cap-dependent translation. The role of mTORC2 in IRES dependent translation cannot be ruled out at this point.
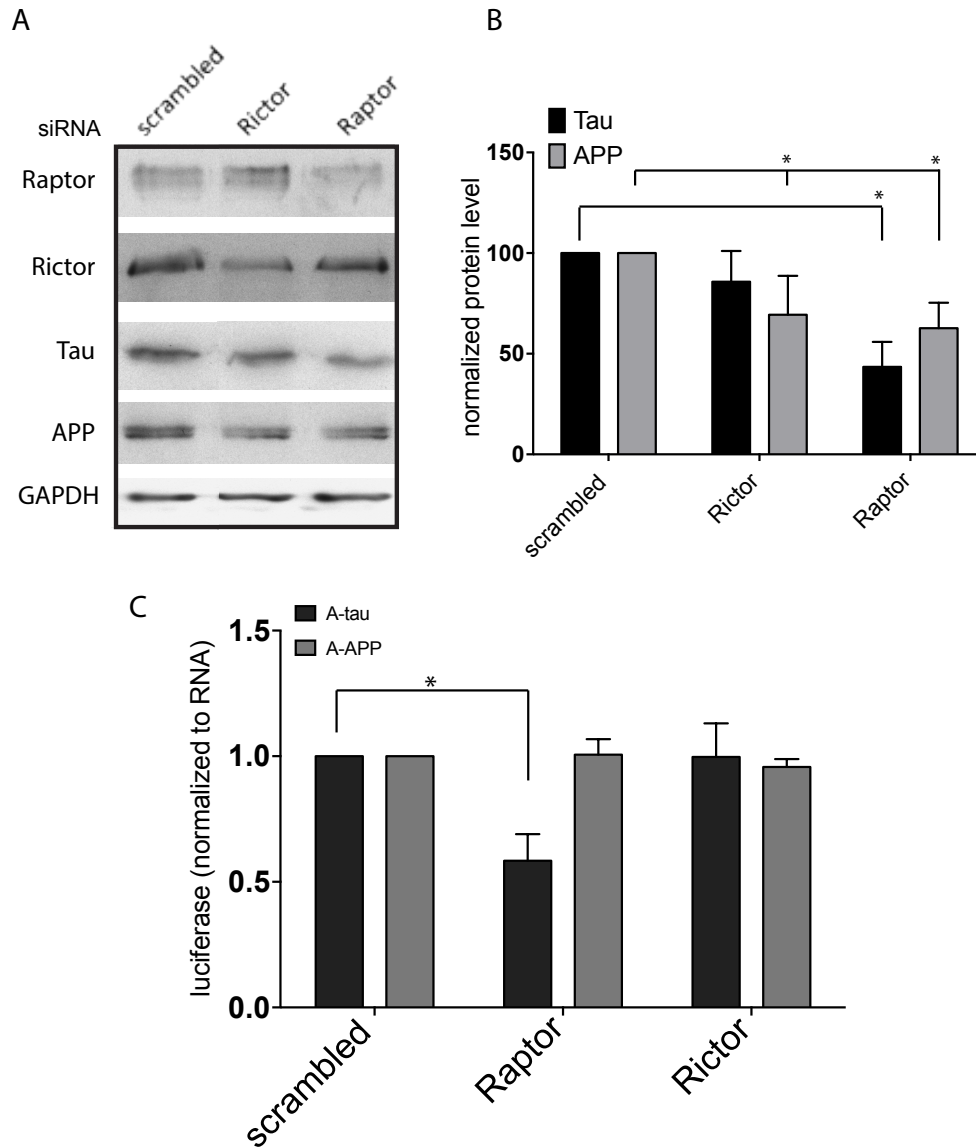
Figure 3.13. **Tau protein expression and IRES dependent translation is reduced by siRNA mediated knockdown of Raptor protein.** SiRNAs to Raptor, or Rictor proteins were transfected into Daoy cells as described. Lysates were analyzed for levels of Raptor, Rictor, Tau, APP, and GAPDH proteins using western blotting (A). Level of tau, and APP proteins (black, and gray bars respectively) were quantitated, normalized to GAPDH levelsand compared to scrambled siRNA treated samples (B). Raptor and Rictor knocked down cells were transfected with ApppG capped *Photinus* luciferase mRNA containing either tau or APP leaders. Samples were then either used in a luciferase assay, or harvested for RT-PCR. A ratio of Photinus luciferase activity to level of *Photinus* RNA for the scrambled siRNA treated cells was set to 1, and the ratios obtained frpm the Raptor and Rictor siRNA treated cells were normalized to the scrambled siRNA treated control (C). (n=3). Error bars represent SD. *p<0.05.

Raptor and Rictor are proteins that are involved in many important cellular processes, including protein synthesis. Prolonged reduction of these proteins could have unidentified effects, and even induce cellular apoptosis. Since protein synthesis is a dynamic process, and I was not able to achieve complete knockdown of mTOR targets, using pharmacological inhibitors to acutely and transiently inhibit these complexes may provide a better understanding of how these complexes regulate IRES mediated translation.

3.4B Tau protein expression is not affected by mTOR inhibition

mTOR inhibitors has been shown to effectively block cap-dependent translation. For example, PP242 is potent active site inhibitor of mTOR that inhibits the activity of both mTORC1 and mTORC2 (Hsieh et al., 2012). I confirmed the efficacy of the drug in Daoy cells through the use of a puromycin incorporation assay. Puromycin resembles the aminoacyl-transfer RNA (aminoacyl-tRNA) and can be incorporated into elongating polypeptide chains by forming a peptide bond (Goodman & Hornberger, 2013; Schmidt, Clavarino, Ceppi, & Pierre, 2009). Puromycin incorporation was visualized by western blotting using anti-puromycin antibody, which is shown in Figure 13A. Puromycin incorporation was readily observed as a smear of bands representing newly synthesized polypeptides, while nothing was detected in the lane with lysate from untreated cells (Figure 3.14 A, first lane). Treatment with PP242, however, caused a significant reduction in protein synthesis as shown by diminished levels of puromycin-incorporated peptides (Figure 3.14 A, last lane).
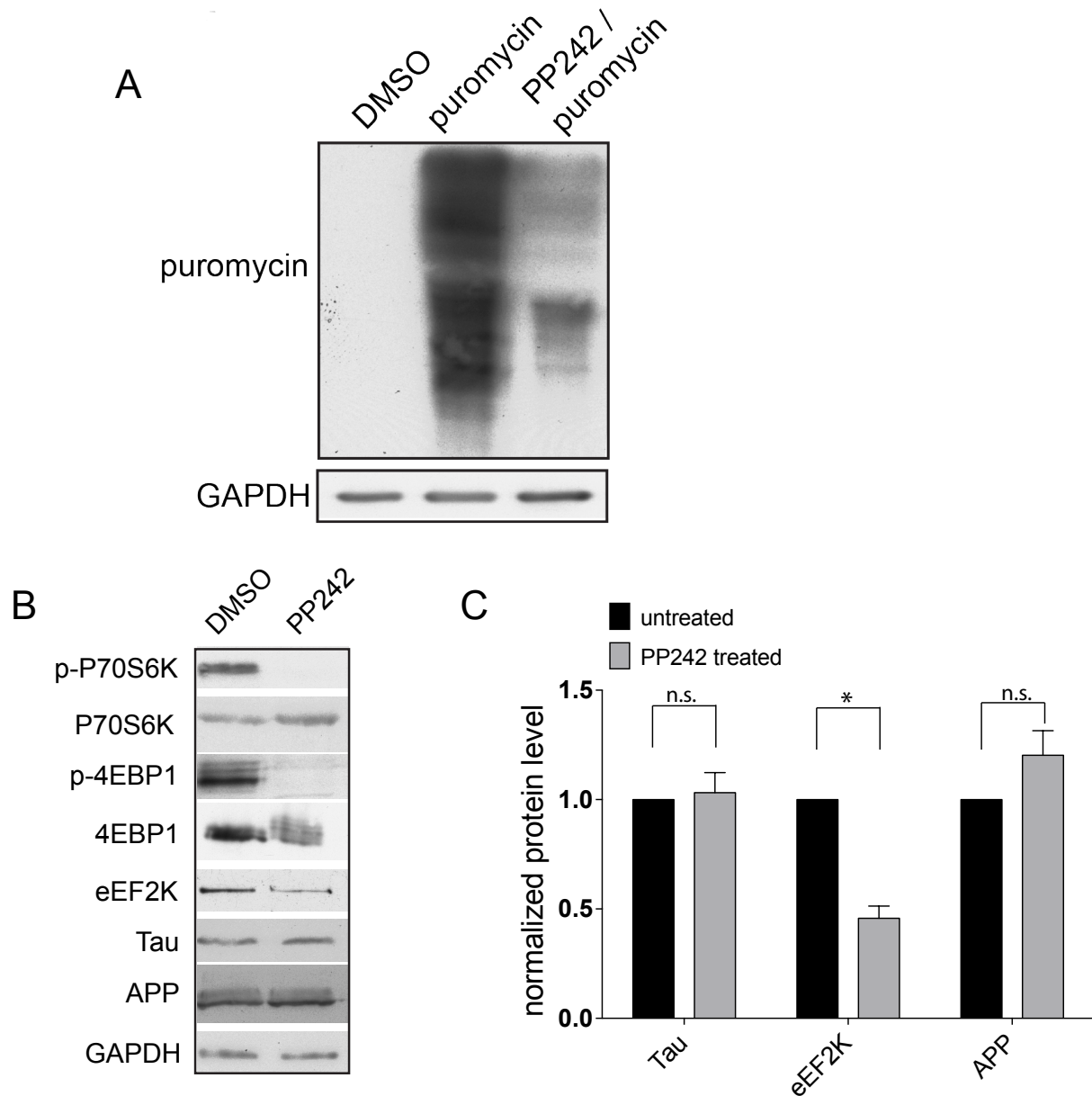
Figure 3.14. **Pharmacologic inhibition of mTOR by PP242 reduces cap-dependent translation.** Daoy cells were either treated with DMSO, or PP242 for three hours. During the last thirty minutes, the medium was supplemented with puromycin as described. (A) The newly synthesized polypeptides incorporated with puromycin were detected using western blotting. (B) PP242 treated cells were analyzed for downstream effectors of mTOR signaling including the phosphorylated p70S6K, and 4EBP1, total levels of p70S6K, 4EBP1, eEF2k, tau, APP, and GAPDH proteins. (C) The level of tau, eEF2K, and APP proteins were quantitated and compared between untreated (black bars) and PP242 treated (gray bars) samples. (n=4). Error bars represent SD. * $p < 0.05$.

In order to confirm that the reduced protein synthesis by PP242 was due to the action of downstream mTOR effectors, lysates from control and PP242 treated Daoy cells were analyzed for the phosphorylation of 4EBP-1 and p70S6K proteins. Western blotting experiments clearly showed that the phosphorylation of both of these proteins was completely abrogated by PP242 (Figure 3.14 B), while the level of total 4EBP-1 and p70S6K proteins did not change. Expression of eEF2K protein was used as a positive control, which was decreased by more than 50% following PP242 treatment (Figure 3.14 B, C). The endogenous expression of tau protein was not affected by mTOR inhibition, while APP protein expression was slightly increased (Figure 3.14 B, C). These results confirm that PP242 could be used to effectively block protein synthesis downstream of mTOR pathway, and that tau, and APP protein expression is not reduced with inhibition of cap-dependent translation.

3.4C Tau protein is continually translated even after inhibition of mTOR

The expression of tau protein remained unaffected even after PP242 treatment. Given the short treatment time (3 hrs) and the estimated half-life of tau mRNA (24 hr) (Sharova et al., 2009) steady protein levels are predicted to be regulated at the levels of synthesis and degradation. To distinguish between these two possibilities I first examined if protein translation was sustained even after mTOR inhibition by PP242. Polysome gradient analysis was performed examine productive association of ribosomes with tau mRNA. This assay utilizes polyribosome fractionation by sucrose density gradient centrifugation to determine the translational efficiency of specific mRNAs.
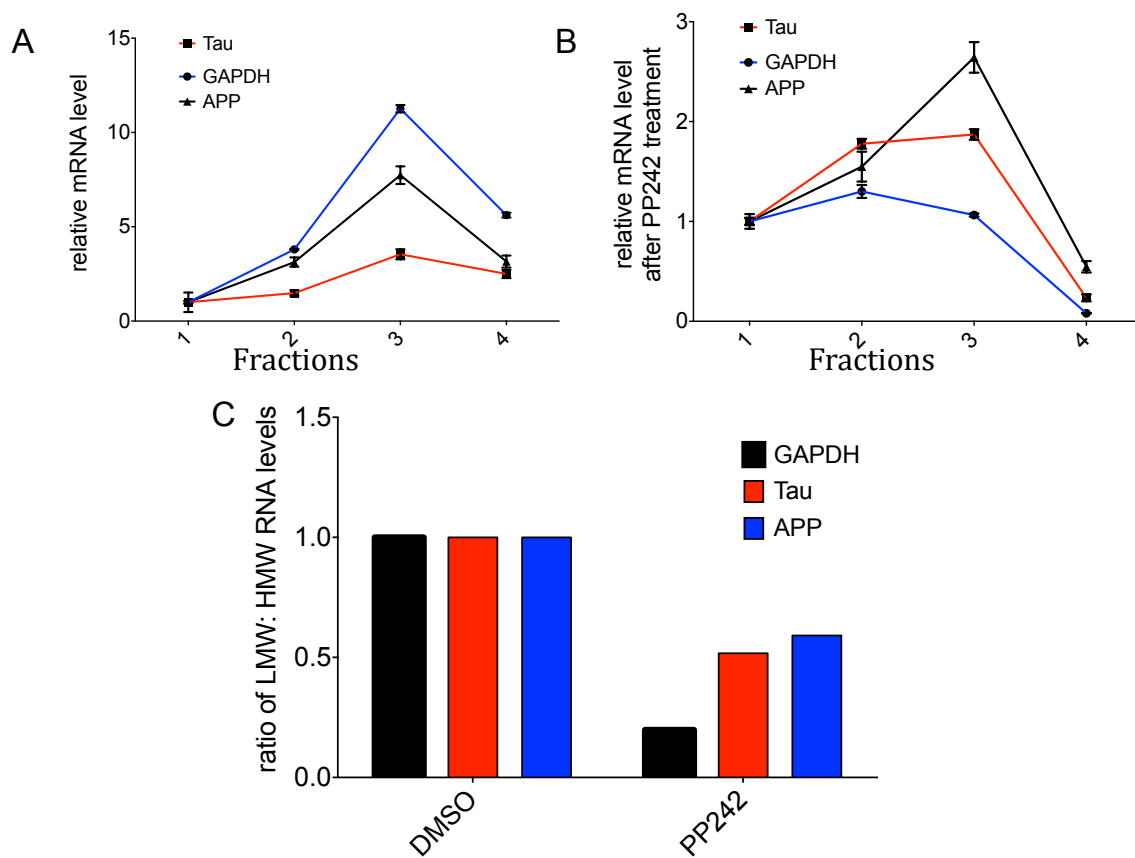
Figure 3.15. **Tau and APP mRNA continue to associate with high molecular weight polysomes after PP242 treatment.** Daoy cells were either treated with DMSO, or PP242 for four hours. Cells were then harvested and then polysome gradient analysis was performed. Total RNA was harvested from fractions representing the non-polysomal (1), low molecular weight (2), and high molecular weight polysomes (3,4). RT-PCR was then performed using tau (red line), APP (black line), or GAPDH (blue line) primers and the values were compared between DMSO treated (A) and PP242 treated (B) cells. (C) A ratio of the mRNAs in the LMW compared to HMW fractions were calculated.

Using this assay, tau mRNA levels were quantified in nonpolysomal, low molecular weight (LMW) polysome and high molecular weight (HMW) polysome fractions between the untreated and PP242 treated cells (See Appendix A1). Association of mRNAs with HMW fractions is indicative of increased translation initiation and/or reinitiation due to efficient loading of the ribosomes onto the mRNA (Thomas & Johannes, 2007). The levels of APP

and GAPDH mRNAs were used as controls. In the untreated cells, majority of the GAPDH, tau, and APP mRNA were associated with HMW fractions, suggesting that these mRNAs were actively translating (Figure 3.15 A). In contrast, there was a great reduction in HMW polysome associated GAPDH mRNA after PP242 treatment (Figure 3.15 B), while tau and APP mRNAs still associated with the HMW fractions. These results indicate that both tau and APP mRNAs continue to be translated even during inhibition of cap-dependent translation by PP242.

3.4D Polysome association of Tau mRNA during mTOR inhibition correlates with upregulated IRES utilization

The expression and active translation of tau protein even after mTOR inhibition with PP242 suggested that IRES dependent translation was utilized for tau protein synthesis. To confirm this, Daoy cells were either left untreated or treated with PP242 for three hours, and then co-transfected with the monocistronic RNA reporters containing tau, or APP leaders as previously described. Cells were then used either for dual luciferase assay or for RT-PCR.

Blocking mTOR activity by PP242 led to a reduction in cap-dependent translation by ~40%, and increased tau IRES activity by more than two fold as measured by the P:RNA ratio (Figure 3.16 A). PP242 also led to an increase in APP IRES activity by ~1.9 fold (Figure 3.16 B). These results suggested that upon inhibition of mTOR signaling (and thus cap-dependent translation), IRES dependent translation is utilized for the translation of tau and APP mRNAs (as shown in Figure 3.15 B, C). Further, since my results showed that both tau and APP IRES activities were upregulated upon mTOR inhibiton, it is likely that specific regulation of these IRESes occur downstream of mTOR signaling, and might involve other pathways.
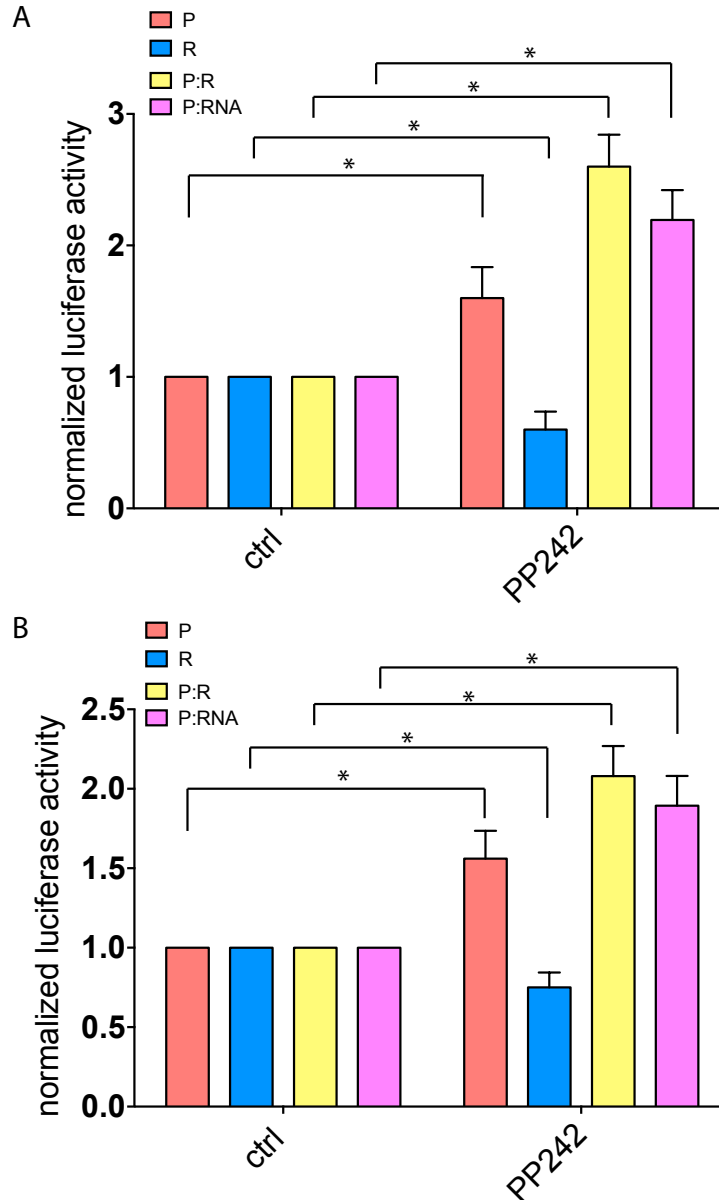
Figure 3.16. **Tau and APP IRES activity is increased following mTOR inhibition by PP242.** Daoy cells were either untreated or treated with PP242 for three hours and then transfected with ApppG capped monocistronic *Photinus* luciferase mRNAs containing the either the tau 5' leader (A), or the APP 5' leader (B) along with m7G capped *Renilla* luciferase mRNA. A comparison of the *Photinus* luciferase (P, red bars), the *Renilla* luciferase (R, blue bars), a ratio of P:R (yellow bars), and a ratio of *Photinus* luciferase activity to *Photinus* RNA level (P:RNA, pink bars) was done for each leader by setting the value obtained from the untreated cells to 1. The luciferase activity for the PP242 treated cells were normalized to the untreated cells. (n=7). Error bars represent SD. * $p < 0.05$.

3.5 SiRNA Library screen for kinases regulating IRES-dependent translation

I showed the MAPK, and the PI3K pathways, as well as mTOR inhibition, are

involved in the regulation of tau IRES. These findings support my hypothesis that tau IRES could be regulated by other pathways as well. In an effort to identify novel pathways, and regulatory networks that regulate the IRES activity of the tau 5' leader, a human kinase siRNA screen was performed in SKNSH cells. The commercially available siRNA library was designed to knockdown a total of 710 kinases with on average 3 siRNA targets per gene (provide information). An average of P:R ratio obtained from three individual experiments is shown in Appendix A2. This screen resulted in four hits – the CHKA, EIF2AK2, PRKX, and ULK2 proteins. CHKA knockdown led to an increase in tau IRES activity, suggesting an inhibitory role for this protein in tau IRES mediated translation. Meanwhile, knockdowns of EIF2AK2, PRKX, and ULK2 reduced tau IRES activity, which suggests that these proteins function to promote the tau IRES.

In order to verify the results from the screen, I picked two proteins from the hits-ULK2 and PRKX, and knocked down the proteins in a separate experiment. Different set of siRNAs were utilized for the knockdown, which resulted in more than 70% reduction of PRKX and 40-50% reduction of ULK2 protein expression (Figure 3.17 A). Unfortunately, I was unable to recapitulate the results from the siRNA screen as reduction of either PRKX, or ULK2 did not affect tau protein expression, or tau IRES dependent translation (Figure 3.17 B, C).
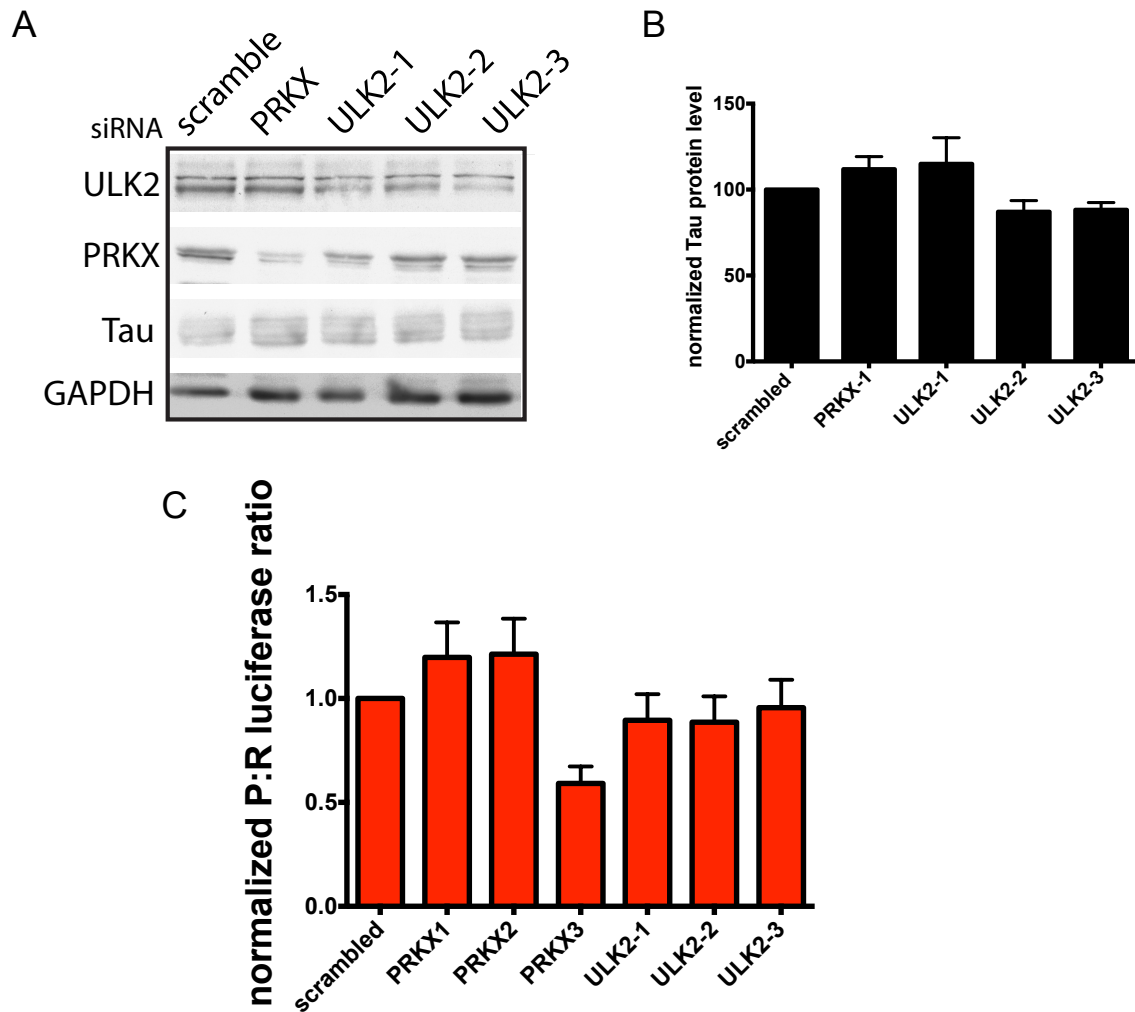
Figure 3.17. **Tau protein expression and IRES dependent translation is unaffected by siRNA mediated knockdown of PRKX and ULK2 proteins.** SiRNAs to Raptor, or Rictor proteins were transfected into Daoy cells as described. Lysates were analyzed for levels of Raptor, Rictor, Tau, APP, and GAPDH proteins using western blotting (A). Level of tau, and APP proteins (black, and gray bars respectively) were quantitated, normalized to GAPDH levelsand compared to scrambled siRNA treated samples (B). Raptor and Rictor knocked down cells were transfected with ApppG capped *Photinus* luciferase mRNA containing either tau or APP leaders. Samples were then either used in a luciferase assay, or harvested for RT-PCR. A ratio of *Photinus* luciferase activity to level of *Photinus* RNA for the scrambled siRNA treated cells was set to 1, and the ratios obtained frpm the Raptor and Rictor siRNA treated cells were normalized to the scrambled siRNA treated control (C). (n=3). Error bars represent SD.

Summary

In this chapter I identified various cellular conditions, signaling molecules and pathways that regulate translation mediated by the tau 5' leader. First, I demonstrated that tau IRES is utilized by many neuronal, and non-neuronal cells during normal cellular conditions. Next, I showed that the tau IRES dependent translation is increased in conditions which is commonly observed in pathologic NDs. Tau IRES activity was upregulated by increased extracellular iron, and dsRNA mimic (poly I:C), as well as by the presence of extracellular Aβ (1-42) peptides. Tau IRES activity was not affected by chemically induced ischemic conditions. However, tau protein expression was not changed during any of these conditions. These results demonstrate that the expression of tau protein is highly regulated. Moreover, these data imply that tau IRES can be utilized to maintain expression of tau protein during normal, and pathogenic cellular conditions.

Next, I attempted to identify signaling molecules and pathways that are involved in regulating tau IRES dependent translation. Since both cap-dependent and IRES dependent pathways utilize some of the same molecules and factors for translation, I examined components of the MAPK, and PI3K-mTOR pathways. Using various growth factors treament, I found that unlike cap-dependent translation, growth factors did not increase translation by the tau IRES. Further, pharmacological inhibition of various kinases in the MAPK and PI3K pathways reduced both cap-dependent and IRES dependent translation. These results further demonstrated that different cellular cues modulate both modes of translation, through similar upstream pathways, likely through the mTOR signaling to regulate translation. Inhibition of mTOR signaling by its active site inhibitor PP242 showed that despite diminishing cap-dependent translation, tau (and APP) proteins were continually

translated, and that during mTOR inhibition, IRES-dependent translation is utilized to maintain tau (and APP) protein expression.

I employed the siRNA screen of kinases to identify novel pathways and networks that regulate tau IRES. Unfortunately, I was unable to repeat the results for two of the four strongest hits (ULK2, and PRKX) from the screen in separate experiments. Different sets of siRNA for the proteins were used between the experiments, which could have resulted in the disparity in the results. Further analysis of the data from the siRNA screen showed that siRNAs to other kinases (such as kinases in the MAPK, and PI3K pathways, that were shown to regulate tau IRES activity) did not affect tau IRES activity. It is possible that the siRNAs in the screen did not completely knockdown the kinases. Further, this screen was performed in SKNSH cells, in the background of normal cap-dependent translation. Performing the screen in other cell lines with better tau IRES usage (such as Daoy, or U87 cells) in normal cellular condition as well as in conditions that increase the tau IRES activity might provide better information regarding regulators of tau IRES.

Chapter IV

Identification and characterization of

cis and trans regulators of tau IRES

For most cellular IRESes, the binding of regulatory factors called IRES trans-acting factors (ITAFs) is thought to be a rate limiting step in initiating translation. ITAFs functioning as enhancers consist of proteins that bind specific sequences in the IRES to either directly recruit the ribosome or to stabilize the secondary structure of the RNA, thereby promoting the binding by other translation initiation factors. Inhibitory ITAFs function to block IRES-mediated translation and potentially down regulate cap-dependent translation. Nearly all ITAFs described to date have recognized roles in RNA processing with members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family being perhaps the most common. Examples of non-hnRNP protein serving as ITAFs include: the La autoantigen (SSB), which has been shown to enhance the XIAP IRES activity by promoting binding of 40S ribosomal subunit (Holcik & Korneluk, 2000); UNR (CSDE1), which promotes p58 PITSLRE IRES (Zhang et al., 2015); HUR (ELAVL), which is a positive ITAF for XIAP IRES, and an inhibitory ITAF for p27 (Kip1), and IGF-1R IRESes (Kullmann, Gopfert, Siewe, & Hengst, 2002). It is important to note that many of the ITAFs are nucleo-cytoplasmic proteins, and their expression and subcellular location are dependent upon cell type and cellular environment. Thus, identification of an ITAF functional role as being specific for a given IRES often can shed light into the cell types and conditions where the IRES is utilized. Finally it is also important to consider the IRES-mediated translation may be more complex than binding of a single protein. There are examples of multiple ITAFs binding to a single IRES, and of ITAFs interacting with proteins bound to additional cis-regulatory sequences present in the 5'- and the 3' UTR that act to modulate IRES dependent translation. In this chapter, I identify novel ITAFs that inhibit tau IRES dependent translation and examine their functional role as well as possible mechanism of this regulation.

4.1 Candidate factor approach for identifying ITAFs for tau IRES

There have been many advances in experimental methodologies to identify proteins that bind to a specific mRNA sequence. A frequently used and unbiased approach is to use mass spectrometry to identify proteins bound to a RNA target sequence either through in vivo expression of a RNA that can be captured or *in vitro* assemble using synthesized RNAs. Unfortunately, previous attempts to perform an unbiased screen for tau IRES-binding proteins, failed to identify any specific proteins. For this reason, I decided to utilize the candidate factor approach for identifying ITAFs with the goal examining the impact of ITAF modulation on IRES-mediated translation of tau mRNA. A summary of the candidate ITAF that were considered along with specific details related to their inclusion is presented in Appendix A3. Four candidate proteins were chosen from the list based on predicted binding sequences located within the tau IRES – the poly C binding protein 2 (PCBP2), the La protein, the polypyrimidine tract binding protein (PTB), and the neural PTB (nPTB). This section experimentally examines the potential role of PCBP2, SSB, PTBP1, and PTBP2 in the regulation of IRES-mediated translation of tau mRNA.

4.1A PCBP2 reduction does not affect tau protein level and IRES activity

PCBP2 is an hnRNP protein that was identified as a poly(C)-binding protein. Its role as an ITAF has been established for both viral and cellular IRESes such as poliovirus, papilloma virus, BAG1, and MYC (Evans et al., 2003). The observation that the tau IRES and 5' UTR sequence contain of several poly (C) stretches (see Figure 4.5 A), provides the rational that PCBP2 might function as a tau ITAF. As a first step I sought to address if PCBP2 might play any role in tau protein expression through knock down experiments.
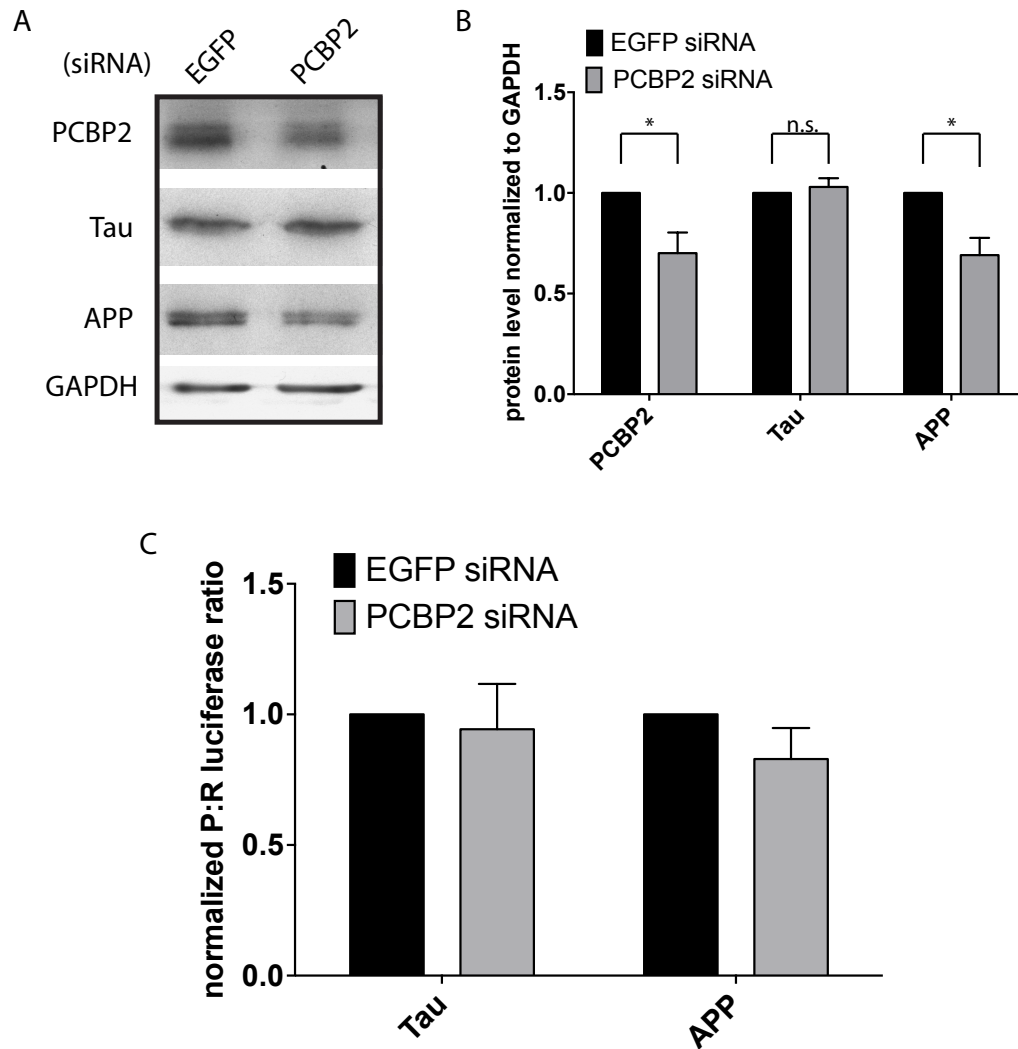
Figure 4.1. **The PCBP2 protein does not function as an ITAF for tau IRES.** U87 cells were transfected with PCBP2, or EGFP control siRNA for 48 hours and western blotting was performed using PCBP2, tau, APP, and GAPDH antibodies (A). (B) The level of PCBP2, tau, and APP proteins were quantitated and normalized to GAPDH levels. (C) ApppG capped monocistronic *Photinus* luciferase mRNA containing either tau, or APP 5' leader were cotransfected into EGFP, or PCBP2 knocked down cells, and m7g capped *Renilla* luciferase mRNA was used as transfection control. P:R ratio was determined and normalized to EGFP siRNA control (n=2). Error bars represent SD.

The human glioma cell line U87 was used as these were the most efficient cell lines

for siRNA transfection. The siRNA targeting PCBP2 was performed for 48 hours, and

lysates analyzed for the level of PCBP2, tau, APP, and GAPDH proteins by western blotting.

A representative western blot is shown in Figure 4.1 A. Quantification of 3 independent

experiments normalized to GAPDH levels is shown in Figure 4.1 B. The results showed an incomplete knockdown of PCBP2 (~40%). PCBP2 reduction was associated a similar decreased APP protein level, however, had no effect on tau protein levels.

I have previously shown that tau IRES dependent translation is influenced by various cellular conditions without observable changes in tau protein level. To investigate whether knockdown of PCBP2 affected tau IRES dependent translation in a similar manner, the siRNA treated cells were examined for tau IRES activity. A ratio of *Photinus* to *Renilla* luciferase activity (P:R) obtained from tau, or APP 5' leader containing mRNAs showed that PCBP2 reduction did not affect tau IRES activity, while it reduced APP IRES activity (~20%) (Figure 4.1 C). These results suggest that PCBP2 may not have a role in regulating the tau IRES, but may function as an ITAF for APP IRES. However, since I was unable to get a complete knockdown of PCBP2, it is possible that the remainder of the protein in the cells is sufficient for tau IRES activity.

4.1B The La protein does not function as an ITAF for tau IRES

The La autoantigen (SSB) also functions as an ITAF for many eukaryotic and viral IRESes. The La protein has been shown to bind the 5'UTR regions of poliovirus, influenza virus, rubella virus, rabies virus, and the vesicular stomatitis virus, likely through a CACAA RNA sequence motif (Duncan & Nakhasi, 1997; Kurilla & Keene, 1983; Meerovitch et al., 1993; Pardigon & Strauss, 1996). The exception is binding of La protein to hepatitis C virus IRES, which has been shown to occur via a GCAC site near the start AUG codon (Pudi, Abhiman, Srinivasan, & Das, 2003; Pudi, Srinivasan, & Das, 2004). The tau 5' leader sequence contains a GCAC sequence, which could serve as a binding site for the La protein. This led us to question whether the La protein functions as a tau ITAF.
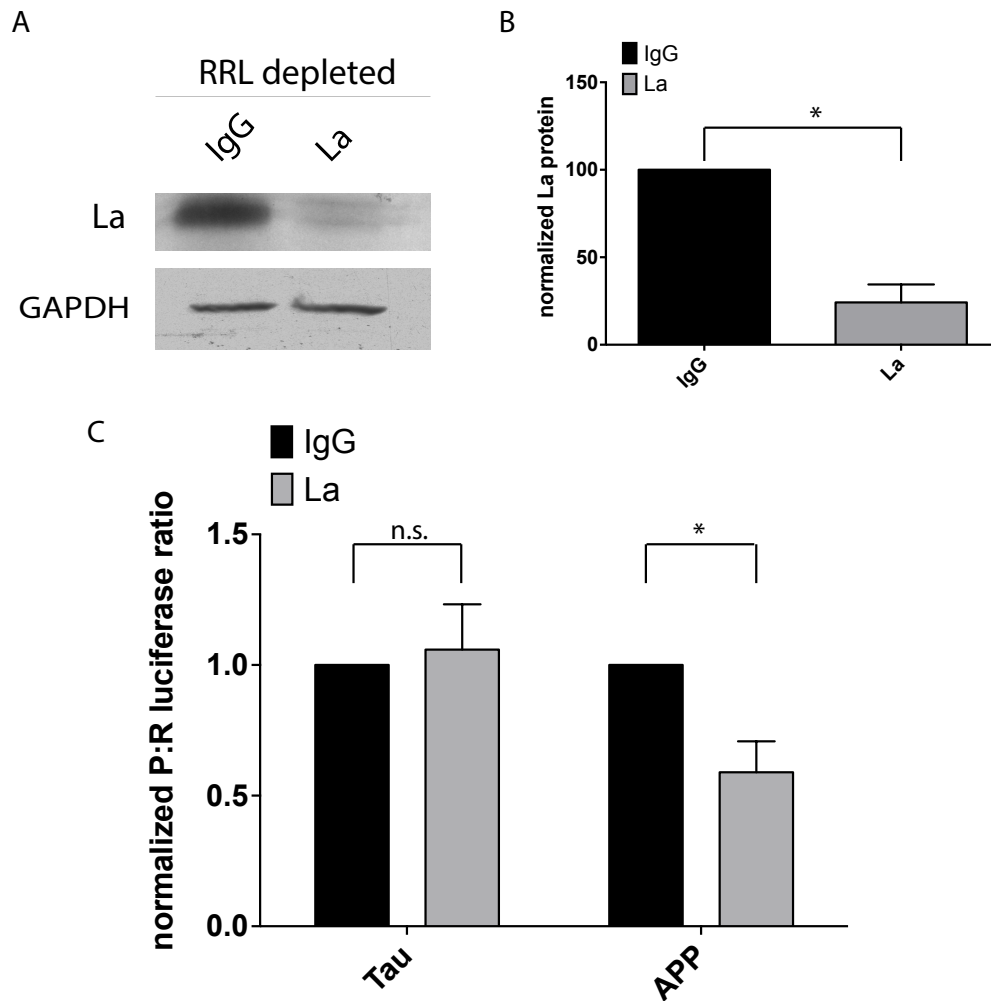
Figuere 4.2. **The La protein does not affect tau IRES activity *in vitro*** (A) Rabbit reticulocyte lysate (RRL) was depleted with La protein (or IgG control) and western blotting was performed to using La and GAPDH antibodies. (B) The level of La protein in IgG antibody treated or La depleted RRL was quantitated and normalized to GAPDH levels. (C) ApppG capped monocistronic *Photinus* luciferase mRNA containing either tau, or APP 5' leader were cotransfected into IgG treated, or La depleted RRL, and m7g capped *Renilla* luciferase mRNA was used as transfection control. P:R ratio was determined and normalized IgG treated lystate (n=3). Error bars represent SD. * $p < 0.04$.

I first addressed this by examining tau IRES activity using La depleted rabbit

reticulocyte lysates (RRL). Antibody mediated depletion of La from the RRL led to a more

than70% reduction of the protein compared to IgG treated control (Figure 4.2 A, B).

Examination of the P:R ratio showed that depletion of La did not affect translation from the

tau IRES containing mRNA, while translation from the APP 5' leader was reduced by almost 40% (Figure 4.2 C). I subsequently tested the functional role of La protein in tau IRES dependent translation in glioblastoma cells using knockdown experiments. For this, U87 cells were transfected with siRNA targeting the La protein for 48 hours. The lysate from the siRNA treated cells was analyzed for the level of La, tau, APP, and GAPDH proteins by western blotting as shown in Figure 4.3 A. I observed a 55% reduction of La protein in the siRNA treated cells compared to scrambled siRNA treated controls (Figure 4.3 A, B). Interestingly, knockdown of La protein did not affect tau protein expression, while APP protein level was decreased by almost 50% (Figure 4.3 B).

To assess whether La plays a role in tau IRES dependent translation, tau IRES activity was examined in cells with La knockdown The P:R ratio from the tau 5' leader containing mRNA was not altered between control and La siRNA treated cells. Conversely, translation from APP 5'leader containing mRNA was reduced by ~40% in La knockdown cells (Figure 3C), which correlate with my previous results in rat glioma C6 cells. Together with the *in vitro* assays in RRL, these results suggest that La protein may not have a functional role as an ITAF for tau IRES, but may function as an ITAF for APP IRES. Indeed, unpublished studies from our lab have shown that La protein binds to the APP 5' leader and enhances APP IRES dependent translation in rat C6 glioma cells.
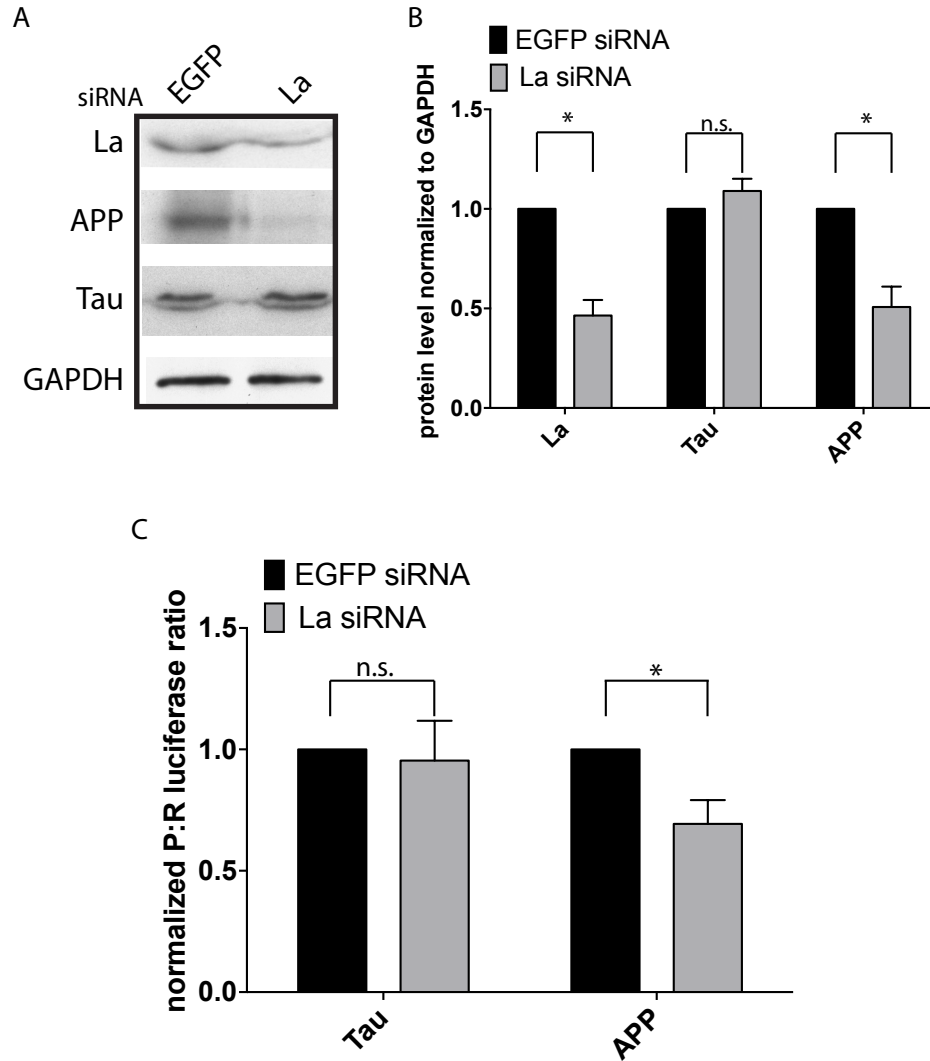
Figure 4.3. **The La protein does not affect tau IRES activity in U87 cells.** (A) U87 cells were transfected with La, or EGFP control siRNA for 48 hours and western blotting was performed using LA, tau, APP, and GAPDH antibodies. (B) The level of La, tau ,and APP proteins were quantitated and normalized to GAPDH levels. (C) ApppG capped monocistronic *Photinus* luciferase mRNA containing either tau, or APP 5' leader were cotransfected into EGFP, or La knocked down cells, and m7g capped *Renilla* luciferase mRNA was used as transfection control. P:R ratio was determined and normalized to EGFP siRNA control (n=2). Error bars represent SD. * p < 0.006.

4.2 Role of PTB and nPTB as inhibitory ITAFs for tau IRES

Polypyrimidine tract binding protein (PTB) is a highly conserved RNA binding

protein known for its role as a regulator of alternative splicing (Wollerton et al., 2004). PTB

functions as a silencer of exon definition for multiple genes such as the FGFR2, GABAAγ2,

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and α-tropomyosin (Sawicka et al., 2008). PTB is ubiquitously expressed, but its expression is downregulated in mature neurons (Boutz et al., 2007; Markovtsov et al., 2000). In the brain, a neuronal homolog of PTB called neuronal PTB (nPTB) is expressed. PTB and nPTB are highly similar proteins and share 74% amino acid sequence similarity (Lillevali, Kulla, & Ord, 2001; Markovtsov et al., 2000). Due to their highly similar sequence homology, PTB and nPTB proteins also have similar cellular functions. In some cases, nPTB has been shown to antagonize the function of PTB and derepress the splicing repression of certain genes such as Rip3 and Exoc1 (Boutz et al., 2007). In addition to their function as splice regulators, PTB and nPTB have additional functions in mRNA stability, RNA localization, translational suppression, and IRES dependent translation of many viral and cellular IRESes (Besse, Lopez de Quinto, Marchand, Trucco, & Ephrussi, 2009; Borovjagin et al., 1994; S. Ma et al., 2007). For the EMCV, PV, and TMEV IRESes, PTB promotes IRES dependent translation by binding the 5'UTR and stabilizing the RNA structure to facilitate recruitment of the ribosome. PTB is also known to bind pyrimidine stretches in a sequence-specific manner, in either the 5'UTR or the 3'UTR acting to bridge RNA sequences, or change the conformation of the RNA structure to facilitate translation efficiency (Clerte & Hall, 2009; Galban et al., 2008). For example, PTB binds to a stem loop in domain H of the EMCV IRES, thereby stabilizing the RNA structure and allowing for ribosome recruitment (Borovjagin et al., 1994).

Both PTB and nPTB proteins are thought to bind similar sequences in the RNA and their consensus binding site ranges from UCUUU to CCUCCU, and CUCU (S. A. Mitchell et al., 2005; Spriggs et al., 2005). Work done by the Willis lab even showed that the (CCU)6 sequence as a hairpin can function as an artificial IRES , which suggests PTB binding to the

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RNA may be sufficient to facilitate internal translation (S. A. Mitchell et al., 2005). In fact, it is speculated that the presence of a polypyrimidine tract is indicative of a viral IRES (Nicholson, Pelletier et al. 1991) (S. A. Mitchell et al., 2005).

In some cases, PTB requires binding of another ITAF to induce the conformational changes required for PTB to bind. The Apaf-1 IRES requires binding by the UNR ITAF followed by PTB or nPTB binding to open the structure for ribosomal recruitment (Sally A. Mitchell et al., 2003). While for some other IRESes, PTB also acts as an inhibitory ITAF. PTB negatively regulates the BiP and UNR eukaryotic IRESs through similar mechanisms (Cornelis et al., 2005; Kim et al., 2000).

The tau IRES sequence consists of three main regions of CU-stretches, which may serve as potential binding sites for PTB or nPTB (Figure 4.5 A). There is a short CU- stretch near the 5'end of the leader, a second short stretch at nucleotides 67-74, and finally a large CU-stretch near the 3' end of the tau leader. Based on the multiple pyrimidine rich regions, I hypothesized that PTB/nPTB may associate with one or more sites within the IRES, and modulate tau IRES activity.

4.2A PTB and nPTB proteins bind to tau leader sequence

In order to establish the role of PTB and nPTB in tau IRES dependent translation, I first examined if PTB and nPTB proteins bind to the predicted sites in the tau 5' leader sequence. RNA immunoprecipitation (RIP) assay was performed on pCMV-tau transfected Daoy cells.(Walker, de Melo Neto, & Standart, 1998). Antibodies to PTB, nPTB, La, and IgG were utilized for the RIP assay. An RT-PCR was then performed using *Photinus* primers, which showed that both PTB and nPTB proteins bound the tau 5' leader sequence

(Figure 4.4, top panel, lane 1 and 2), while La protein did not bind the sequence (Figure 4.4, top panel, lane 3).
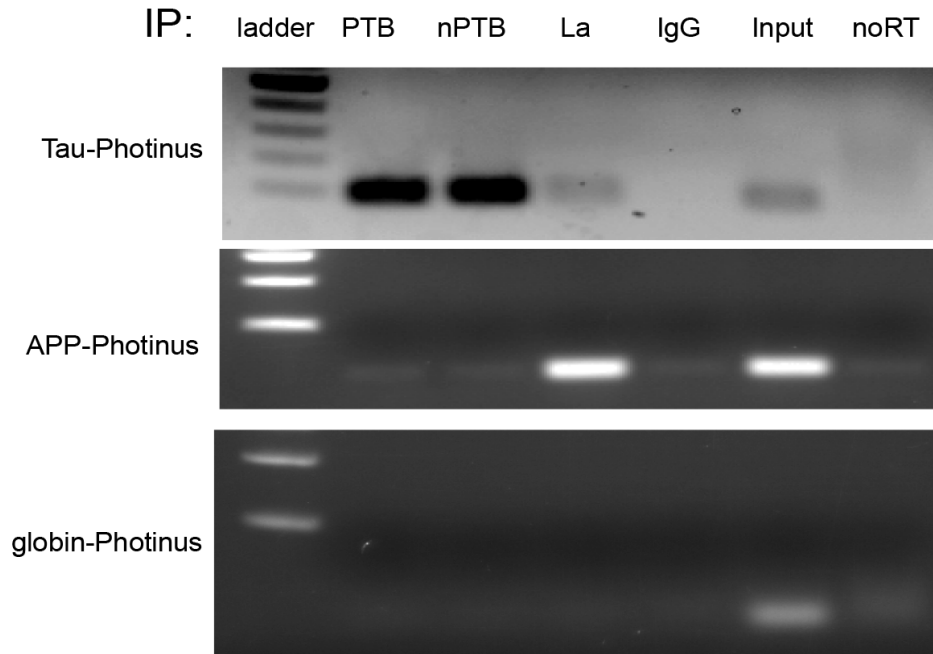
Figure 4.4**. PTB and nPTB proteins bind to tau leader sequence**. pCMV plasmid containing either tau, APP, or β-globin leader upstream of *Photinus* luciferase sequence was transfected into Daoy cells. RNA immunoprecipitation assay was performed using PTB, nPTB, La, or IgG antibodies. Total RNA was harvested and RTPCR was performed using *Photinus* primers. Input (10% of total lysate), and no RT RNA were used as positive and negative controls.

In order to show specificity for the binding, RIP assay was performed using pCMV-APP and pCMV- β-globin transfected cells. Here the RT-PCR analysis showed that La protein bound the APP 5' leader sequence (Figure 4.4, middle panel), which was consistent with previous results from the lab. As expected, the β-globin leader did not associate with PTB, nPTB, or La proteins (Figure 4.4, bottom panel). PTB and nPTB proteins did not bind the APP 5' leader sequence. These data demonstrate that both the PTB

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and nPTB proteins are capable of binding the tau IRES sequence and this binding is specific to the tau 5' leader sequence.

4.2B Mutations in the pyrimidine stretches within the tau 5' leader abolish tau IRES dependent translation

I next sought to determine which sequences in the tau 5' leader are important for binding to PTB and nPTB proteins. The tau leader sequence consists of three major stretches of pyrimidines, which could serve as putative PTB/nPTB binding sites. In order to determine if any or all of these regions are essential for the tau IRES, I generated mutation constructs targeting the three main pyrimidine rich regions within the tau leader- PTB mut1, PTB mut2, and PTB mut3 (Figure 4.5 B). Analysis of tau IRES activity displayed significantly reduced translation by PTB mut2 and PTB mut3 constructs (Figure 4.5 C). Of note the changes introduced into the PTB1 mut1 construct were found to inhibit *in vitro* transcription preventing an analysis of this predicted binding site.

My results suggest that disruption of PTB/nPTB binding by mutating the binding sites in the tau 5' leader reduces tau IRES activity, thus implicating PTB and nPTB as positive ITAFs for tau IRES. However, there are two specific caveats to these findings. First, I did not confirm that the mutations indeed prevented PTB/nPTB binding. And secondly, I cannot rule out that the mutations introduced altered the secondary structure of the IRES such that the interaction of other ITAFs was altered. We have previously shown that mutations or small deletions that disrupt the secondary structure of the tau 5' leader RNA completely abrogate tau IRES dependent translation (Veo & Krushel, 2012). Therefore, the above experiments using the mutated sequences is not sufficient to examine the role of PTB/nPTB

as ITAFs for the tau IRES. Loss of function, and/or gain of function experiments are required

to further understand the role of PTB and nPTB proteins in tau internal translation.

A **CCTCCCCT**GGGGAGGCTCGCGTTCCCGCTGCTCGCGCCTGCGCCGCCCGCCGGCCTCAGGAACGCG
CCCTCTTCGCCGGCGCGCGCCCTCGCAGTCACCGCCACCCACCAGCTCCGGCACCAACAGCAGCGC
CGCTGCCACCGCCCACCTTCTGCCGCCGCCACCACAGCCA**CCTTCTCCTCCTCC**G**CTGTCCTCTCCC**G
TCCTCGCCTCTG**TC**GACTATCAGGTGAA**CTTT**GAACCAGG

B Tau240 / PTBmut1 / PTBmut2 / PTBmut3

C
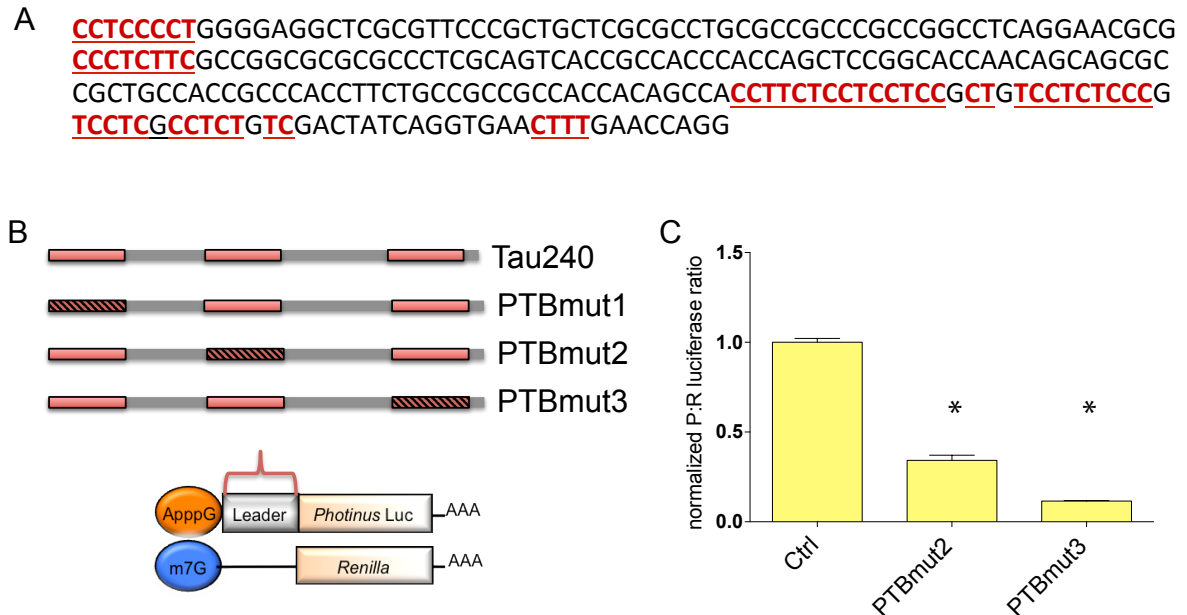
Figure 4.5. **Mutations in the pyrimidine stretches within the tau 5' leader abolish tau IRES dependent translation.** (A). Sequence of the MAPT 5' UTR with the putative PTB binding sites marked in red. (B)A schematic showing the wild type tau 5' UTR construct (tau 240) and the constructs with mutations in the PTB binding sites (PTB mut1, PTB mut2 and PTB mut3). The solid red box indicates wildtype sequences while the shaded red box indicates mutated sequences. (C) ApppG capped *Photinus* luciferase mRNA with tau 240, PTB mut2, or PTB mut3 leaders were co-transfected with m7g capped *Renilla* luciferase mRNA. P:R ratio was determined and normalized to the value obtained from mRNA with tau 240 leader (n=3). Error bars represent SD. * $p < 0.0005$.

4.2C PTB depletion enhances tau IRES activity *in vitro*

In order to establish if PTB is required for tau IRES mediated translation I

examined the impact of depletion using RRL *in vitro* translation experiments. PTB, or

eIF4G1 proteins were depleted from rabbit reticulocyte lysates (RRL) using antibody

conjugated magnetic protein G beads as described. Antibody mediated depletion led to 60%

reduction of PTB, and 80% reduction of eIF4G1 protein compared to IgG only treated lysate

which is shown in Figure 4.6 A. *In vitro* translation assay demonstrated an increase in

luciferase activity from tau 5' leader in PTB depleted lysates compared to IgG treated lysates (Figure 4.6 B, red bars). This result suggests that PTB functions as an inhibitory factor for the tau IRES rather than an enhancer. PTB depletion did not alter translation from mRNAs containing AuroraA (Figure 4.6B, pink bars) or APP leaders (Figure 4.6 B, blue bars). As a positive control, EMCV 5'leader containing *Photinus* luciferase mRNA was used, which was previously shown to be stimulated by binding of PTB (Borovjagin et al., 1994). As predicted, translation from EMCV IRES containing mRNA was reduced by almost 50% in PTB depleted RRL lysates compared to IgG treated control (Figure 4.6 B, orange bars).

Next, I wanted to determine whether the scaffolding protein eIF4G1 is required for tau IRES dependent translation. EIF4G1 is essential in cap-dependent translation to bring different components of the translation initiation machinery on to the mRNA. During infection, many viruses cleave eIF4G1 such that it is not able to bind the cap binding protein eIF4E, but is still able to recruit rest of the translational machinery. This mechanism inhibits global cap-dependent translation. However, the role of eIF4G1 protein in IRES mediated translation of mRNAs is not well understood. It is thought that the eIF4G1 protein is capable of initiating internal translation of IRES containing mRNAs. This is because the related protein, eIF4G2 or DAP5, which is very similar to the cleaved eIF4G1 protein, has been shown to required for IRES mediated translation of many mRNAs, particularly during conditions of ER stress, apoptosis, and mitosis (Henis-Korenblit et al., 2002; Lewis et al., 2008; Marash et al., 2008). Results from *in vitro* translation assay showed that translation from all the leaders tested (tau, APP, AuroraA, and EMCV) was reduced in eIF4G1 depleted RRL compared to IgG treated lysates (Figure 4.6 B). These results showed that eIF4G1 is required for the internal translation mediated by these IRESes.
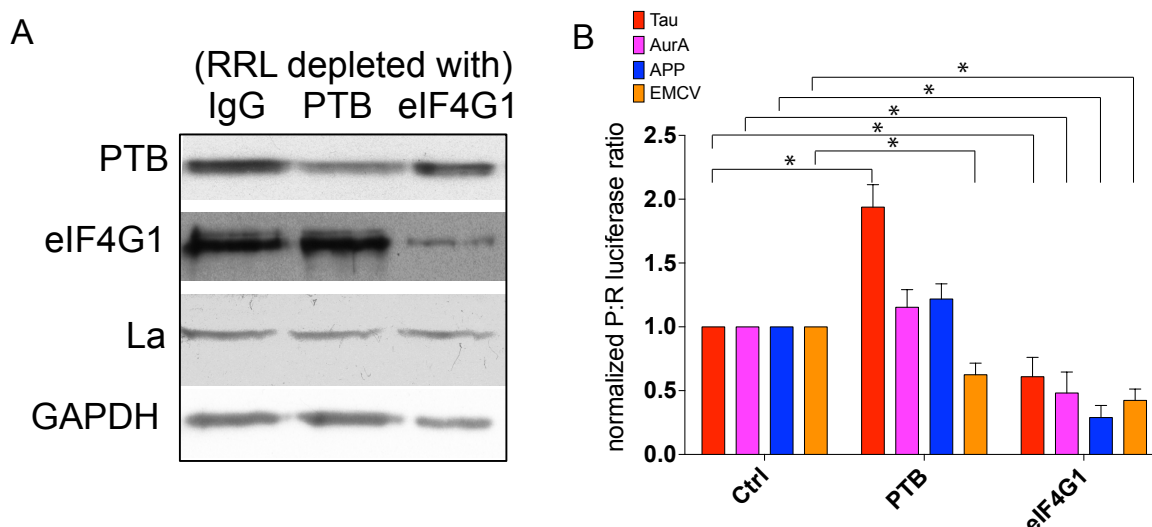
Figure 4.6. Depletion of PTB, or eIF4G1 decreases tau IRES activity in rabbit reticulocyte lysates (RRL). Micrococcal nuclease treated RRL were treated with IgG, PTB, or eIF4G1 conjugated protein G beads and the lysate was used in western blotting to detect the level of PTB, eIF4G1, La, and GAPDH proteins (A). (B) IgG control or PTB, or eIF4G1 depleted cells were cotransfected with ApppG capped *Photinus* luciferase with either tau (red bars), AurA (pink bars), APP (blue bars), or EMCV (orange bars) leaders along with m7G capped *Renilla* luciferase mRNA. P:R ratio was determined and normalized to the value obtained from IgG treated lysates (n=4). Error bars represent SD. * $p < 0.0004$.

4.2D Addition of exogenous nPTB reduces tau IRES dependent translation

The nPTB protein is selectively expressed in mature neurons and muscle cells. In these cells, nPTB expression is preceded by reduced expression of PTB. In RRL, nPTB protein is not expressed. Therefore, to understand its role in tau IRES mediated translation I employed a gain of function assay through the addition of recombinant nPTB to the *in vitro* translation assay. The addition of exogenous nPTB decreased tau IRES activity in a dose dependent manner as shown in Figure 4.7A. The highest concentration of nPTB (1μM) led to more than 70% decline in tau IRES activity. Recombinant GST was used as a control, which did not affect translation by the tau IRES. As, a control for specificity, APP 5'leader containing mRNA was used. Addition of nPTB did not alter APP IRES mediated translation

(Figure 4.7 B). These results imply that both PTB and nPTB proteins have similar inhibitory roles preventing the use of the tau IRES.
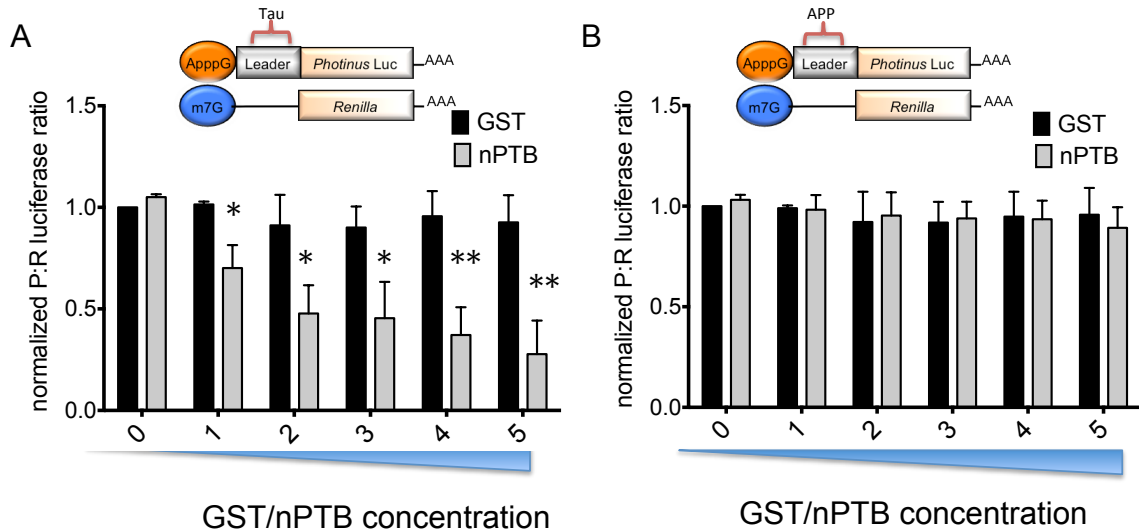
Figure 4.7**. Addition of exogenous nPTB reduces tau IRES dependent translation.** 0.0001-1µM of recombinant human nPTB (gray bars), or GST (black bars) was added to RRL. *In vitro* translation assay was then performed using ApppG capped *Photinus* luciferase mRNA with tau (A), or APP (B) leaders. m7G capped Renilla luciferase mRNA was added as a control. P:R ratio was determined and normalized to the value obtained from untreated lysates (n=3). Error bars represent SD. * $p < 0.007$, ** $p < 0.001$.

4.2E Knockdown of PTB increases endogenous tau protein expression and tau IRES dependent translation

Next, I sought to investigate if PTB, and nPTB inhibit tau IRES mediated translation in neural cells. As a first step, I examined the effect of PTB knockdown on endogenous tau protein expression. The human medulloblastoma cells, Daoy, cells were utilized because of previously shown PTB, but not nPTB expression. The siRNA targeting PTB was performed for 48 hours. A representative western blot demonstrating expression of PTB, nPTB, APP, La, UNR, and GAPDH proteins is shown in Figure 4.8 A.
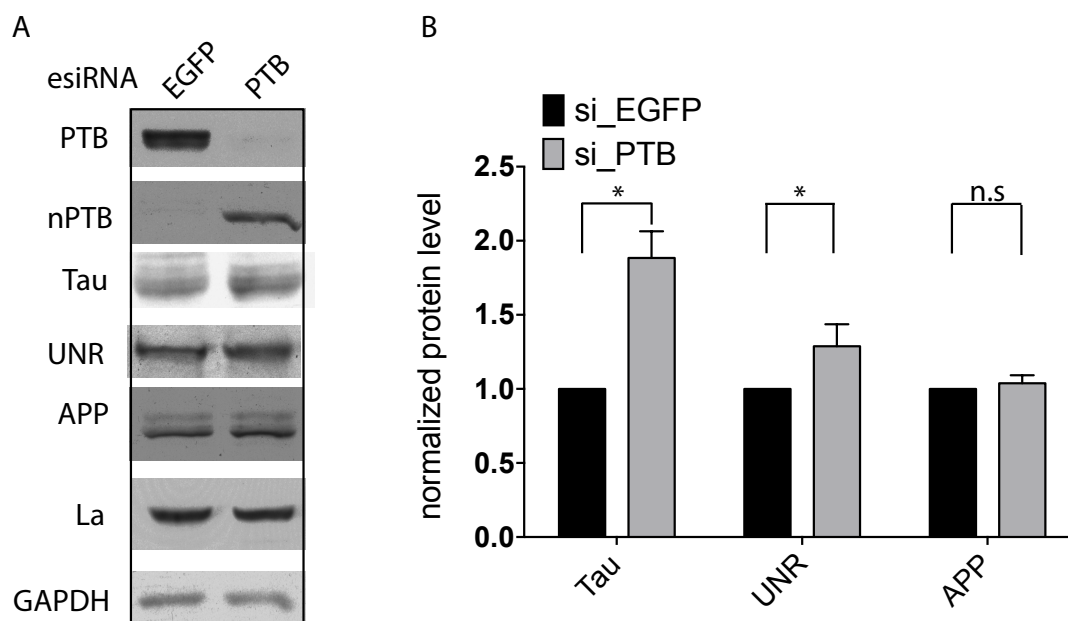
Figure 4.8. **Knockdown of PTB increases endogenous tau protein expression.** Daoy cells were treated with EGFP or PTB siRNA for 48 hours. (A) Western blotting was performed using PTB, nPTB, tau, UNR, APP, La, or GAPDH antibodies. (B) The level of tau, UNR, and APP proteins in PTB siRNA treated cells (gray bars) and EGFP siRNA control treated cells (black bars) was quantitated and normalized to the level of GAPDH (n=4). Error bars represent SD. * $p < 0.03$.

Treatment with siRNA led to a more than 90% reduction in PTB expression (Figure 4.8 A). UNR protein expression was examined as a control. PTB negatively regulates UNR IRES dependent translation (Cornelis et al., 2005), but its affect on UNR protein level is not known. PTB reduction led to a small increase in UNR protein level, but did not affect APP, or La protein expression. (Figure 4.8 A, B). Conversely, PTB knockdown increased tau protein expression by 80% (Figure 4.8A, B).

In cells, levels of PTB and nPTB are highly controlled. PTB autoregulates its expression as well as regulates the expression of nPTB (Boutz et al., 2007; Spellman et al., 2007). PTB induces removal of nPTB exon 10, which introduces a premature stop codon (PTC) causing nonsense mediated decay (NMD) of the nPTB mRNA (Spellman et al., 2007). Consequently, I observed elevated nPTB expression in the PTB knockdown cells. Since I

demonstrated that nPTB binds tau mRNA, it is possible that the increased tau protein levels seen in PTB knockdown cells is due to the induced expression of nPTB and not due to reduction of PTB. In order to distinguish the roles of PTB and nPTB, I performed a double knockdown using siRNA to both PTB and nPTB. Knocking down both PTB and nPTB still increased tau levels compared to nonsense siRNA control (Figure 4.9 A, B). Since the levels of nPTB expressed in the cells is very minimal in comparison to PTB, using nPTB siRNA alone did not affect tau protein expression. These results confirm that reduction of PTB cause derepression of tau, thereby elevating tau protein levels.

I next tested whether PTB reduction increases tau IRES dependent translation in the cells. Similar to the results from the *in vitro* translation assay, of tau IRES was increased by 1.8 fold, and 1.65 fold respectively in PTB, and PTB/nPTB double knockdown cells respectively (Figure 4.9 C, red bars). The single and double knockdown of PTB, and PTB/nPTB reduced translation mediated by the positive control, EMCV IRES by almost half (Figure 4.9 C, orange bars), while translation by the APP IRES was not affected (Figure 4.9 C, blue bars). These results further corroborate the inhibitory function of PTB in tau IRES dependent translation.
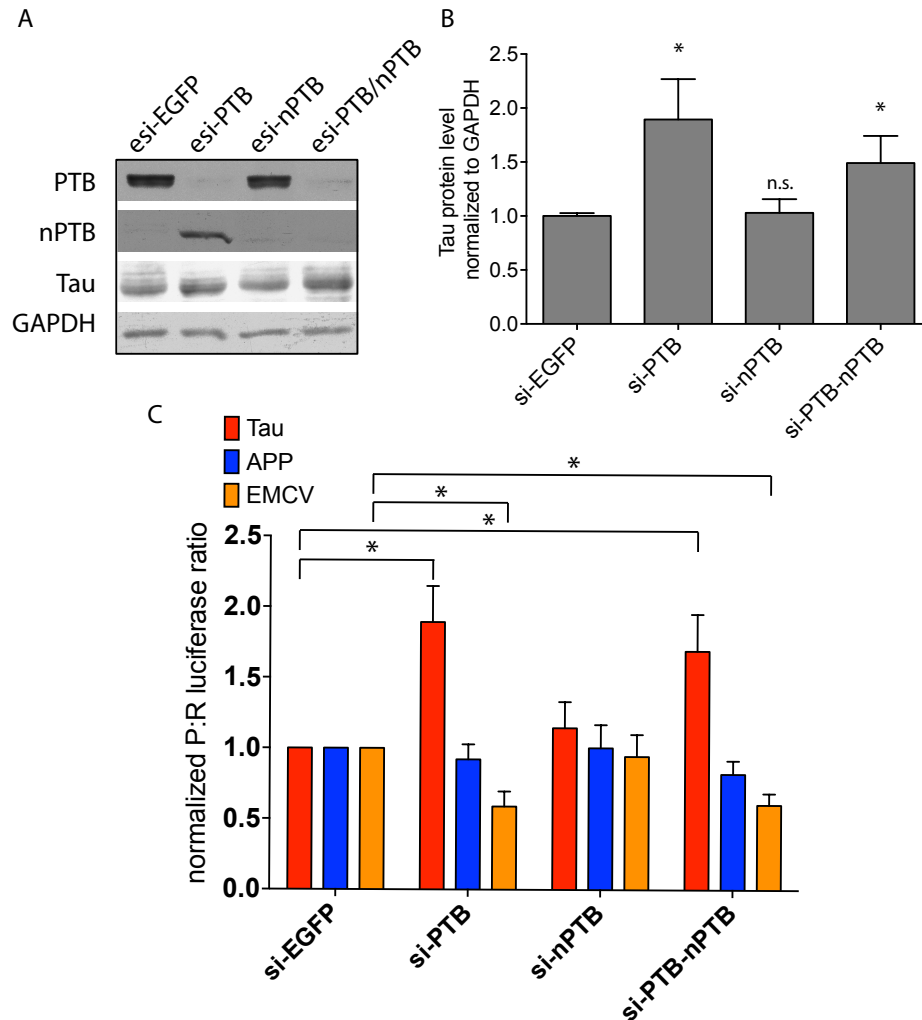
Figure 4.9. **Knockdown of PTB and nPTB increases endogenous tau protein expression and IRES activity.** Daoy cells were treated with EGFP, PTB, nPTB, or PTB and nPTB siRNA for 48 hours. (A) Western blotting was performed using PTB, nPTB, tau, and GAPDH antibodies. (B) The level of tau protein in the knocked down cells was quantitated and normalized to the level of GAPDH. (C) ApppG capped *Photinus* luciferase mRNA with either tau (red bars), APP (blue bars), or the positive control EMCV (orange bars) leaders were cotransfected with m7G capped *Renilla* luciferase mRNA into the siRNA treated cells. P:R ratio was determined and normalized to the value obtained from EGFP siRNA treated cells (n=6). Error bars represent SD. * p < 0.008.

4.2F Overexpression of PTB, and nPTB reduces tau protein expression and IRES

activity

In the absence of a good neuronal cell model to examine the potential role of nPTB

in tau IRES activity I overexpressed nPTB using lentiviral vectors. Among the cell lines I

had shown to have tau IRES use stable nPTB expression was only achieved in U87 cells.

Along with nPTB, I also overexpressed PTB, as well as both PTB/nPTB. Using the lentiviral

vectors, I was able to increase the expression of PTB by 1.5 fold, and get a strong expression

of nPTB. A representative western blot displaying the levels of PTB, nPTB, tau, and GAPDH

is shown in Figure 4.11 A.
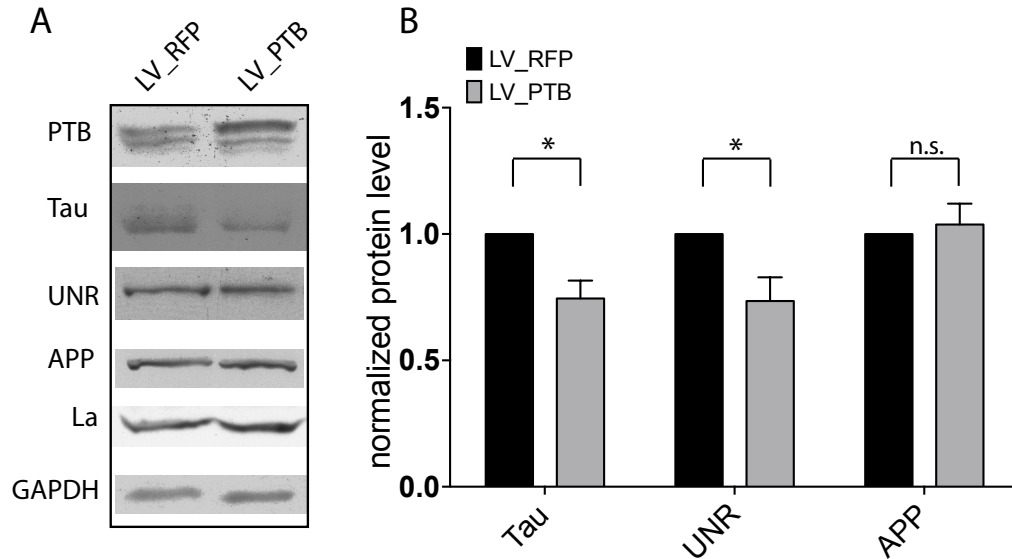
Figure 4.10. **Overexpression of PTB reduces endogenous tau protein expression.** U87 cells were transduced with RFP or PTB lentivirus (MOI=5) for 72 hours. (A) Western blotting was performed using PTB, nPTB, tau, UNR, APP, La, or GAPDH antibodies. (B) The level of tau, UNR, and APP proteins in PTB overexpressing cells (gray bars) was quantitated and normalized to GAPDH levels (black bars) (n=3). Error bars represent SD. * p < 0.008.

Overexpression of nPTB decreased tau protein levels by 20% (Figure 4.11A, B).

Moreover, consistent with previous experiments overexpression of only PTB, or both

PTB/nPTB reduced tau expression (25% and 23% respectively). Interestingly,

overexpression of nPTB reduced PTB protein level as well (Figure 4.11A), suggesting that

the expression of PTB and nPTB is highly controlled, and perhaps nPTB also regulates the

expression of PTB likely through NMD pathways. However, further investigation is required

to fully define the role of nPTB in PTB protein expression. As a positive control, I examined

the expression of UNR protein in PTB overexpressing cells. UNR expression was reduced by

PTB overexpression (Figure 4.10 A, B). However, PTB overexpression did not affect APP,

or La protein levels (Figure 4.10 A, B).
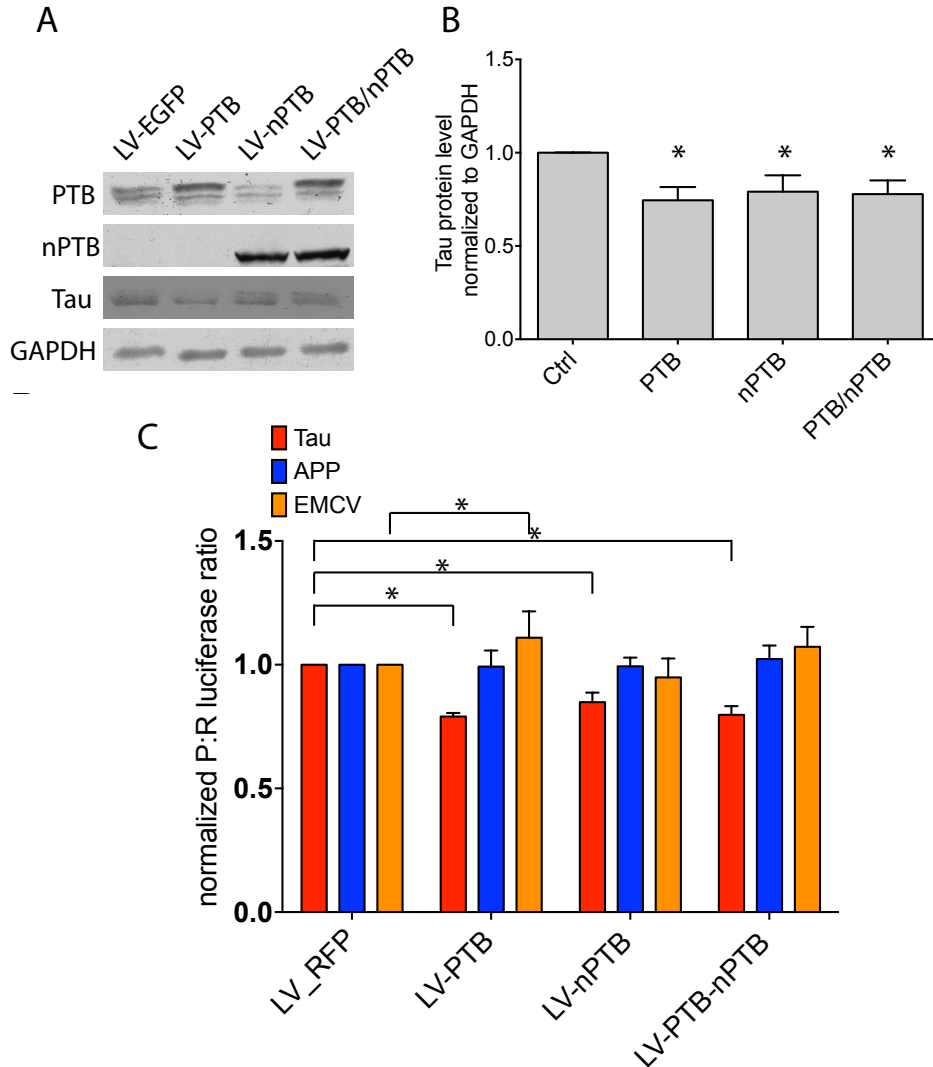
Figure 4.11**. Overexpression of PTB and nPTB increases endogenous tau protein expression and IRES activity.** U87 cells were transduced with RFP, PTB, nPTB, or PTB and nPTB lentivirus (MOI=5) for 72 hours. (A) Western blotting was performed using PTB, nPTB, tau, or GAPDH antibodies. (B) The level of tau protein in the overexpressign cells was quantitated and normalized to GAPDH levels. (C) ApppG capped *Photinus* luciferase mRNA with either tau (red bars), APP (blue bars), or the positive control EMCV (orange bars) leaders were cotransfected with m7G capped *Renilla* luciferase mRNA into the PTB, nPTB, or PTB and nPTB overexpressing treated cells. P:R ratio was determined and normalized to the value obtained from RFP lentivirus treated control (n=4). Error bars represent SD. * p < 0.05.

Finally, I examined tau IRES dependent translation in overexpressing cells. Overexpression of nPTB, PTB, or both nPTB and PTB decreased tau IRES activity by ~20, ~30 and ~30% respectively compared to control cells (Figure 4.11 C, red bars), while APP IRES dependent translation was not affected (Figure 4.11 C, blue bars). Interestingly, overexpression of either nPTB PTB, or both did not affect EMCV IRES activity. The level of PTB protein in U87 cells is already elevated compared to other non-cancerous cells. It is possible that maximum EMCV IRES dependent translation is achieved in U87 cells without having to overexpress PTB, thus masking the effect of PTB overexpression on the IRES activity. Consequently, due to already high level of PTB protein in U87 cells the siRNA experiments provide the best measure of PTB's role in IRES regulation in these cells.

4.3 Role of PTB and nPTB in regulating tau IRES in different cellular conditions

The results so far have demonstrated that PTB and nPTB proteins associate with and repress tau translation through its IRES. Our knowledge of other IRESes and their corresponding ITAFs show that IRES mediated translation is dependent on the expression, and subcellular localization of the ITAF. The upregulation of a specific IRES dependent translation is often times preceded by induced expression, or translocation of an ITAF. Experiments shown in this section examine whether the expression of the newly identified tau ITAF PTB is altered in cellular conditions in which tau IRES dependent translation is upregulated.

4.3A Increased iron, and poly (I:C) treatment do not affect PTB protein expression

Results from experiments presented in the previous chapter showed that increased extracellular iron by FAC treatment as well as treatment with a dsRNA mimic poly (I:C) increased tau IRES dependent translation. Despite the increase in tau IRES mediated

translation, the endogenous level of tau protein was not affected by the addition of FAC or

poly (I:C). Studies have shown that the expression of PTB is reduced during oxidative stress

following H2O2 treatment (Cote et al., 2012) providing support for a potential link with

regulation of IRES use. This led us to question whether the observed during increased iron,

or poly (I:C) treatment was due to altered expression of PTB under these conditions. Lysates

from the FAC and poly (I:C) treated SKNSH cells were examined for PTB expression, which

showed that PTB protein expression was not affected by treatment with FAC, or poly (I:C)

(Figure 4.12 A, B).
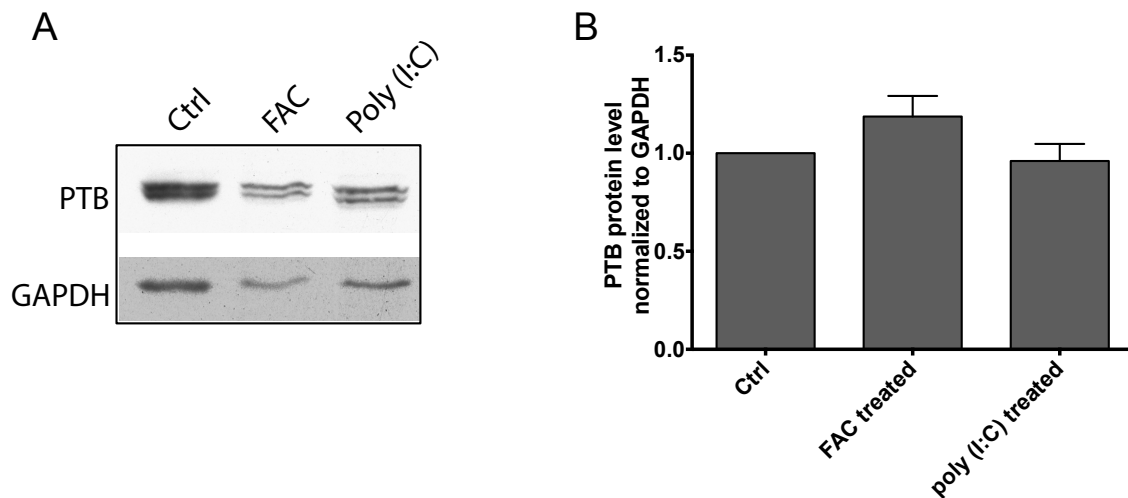
Figure 4.12. **PTB protein expression is not affected by FAC, or poly (I:C) treatment.**
SKNSH cells were treated with either DMSO, ferric ammonium citrate (FAC) supplemented
media for 24 hours, or 500 ug/mL of poly (I:C). (A) Western blotting was performed to
detect PTB and GAPDH protein levels in untreated and FAC, or poly (I:C) treated cells. (C)
The level of PTB protein was quantitated and normalized to GAPDH

This result suggests that the observed increase in tau IRES activity after increased

iron, or poly (I:C) addition is not due to reduced level of PTB protein. The findings do not

rule out that the increase in tau IRES activity during these conditions is not dependent upon

PTB. It is possible that these conditions affect PTB subcellular localization, or disrupt PTB binding to the tau IRES without affecting PTB expression, thus allowing the IRES dependent translation to occur. It is also possible that these conditions increase the expression of yet unidentified tau ITAFs, which could then bind the tau IRES and result in enhanced IRES activity.

4.3B PTB protein expression is differentially affected by inhibitors of kinases in the PI3K and MAPK pathways

In the previous chapter, I demonstrated that inhibiting various kinases in the PI3K and MAPK signaling pathways reduces tau IRES dependent translation. My next step was to examine PTB protein expression after inhibition of these kinases. I again used Daoy cells treated with inhibitors of MEK1/2 , PI3K, Akt, JNK and p38MAPK kinases (Figure 4.13 A). Western blot analysis revealed that inhibition of MEK1/2, JNK, and p38 MAPK kinases increased PTB levels by 1.3, 1.4, and 1.7 fold respectively, while PI3K and Akt inhibitors did not affect PTB expression (Figure 4.13 A,B).

The analysis of tau IRES activity in these conditions showed that JNK, p38MAPK, Akt, and PI3K inhibitors reduced tau IRES dependent translation, while MEK 1/2 inhibitor did not affect tau IRES activity. These results cannot be fully explained by the changes in PTB protein levels caused by these inhibitors though again subcellular localization was not examined. The data suggest that other factors (likely other ITAFs and initiation factors) are involved, perhaps along with PTB, in modulating tau IRES dependent translation in these conditions.
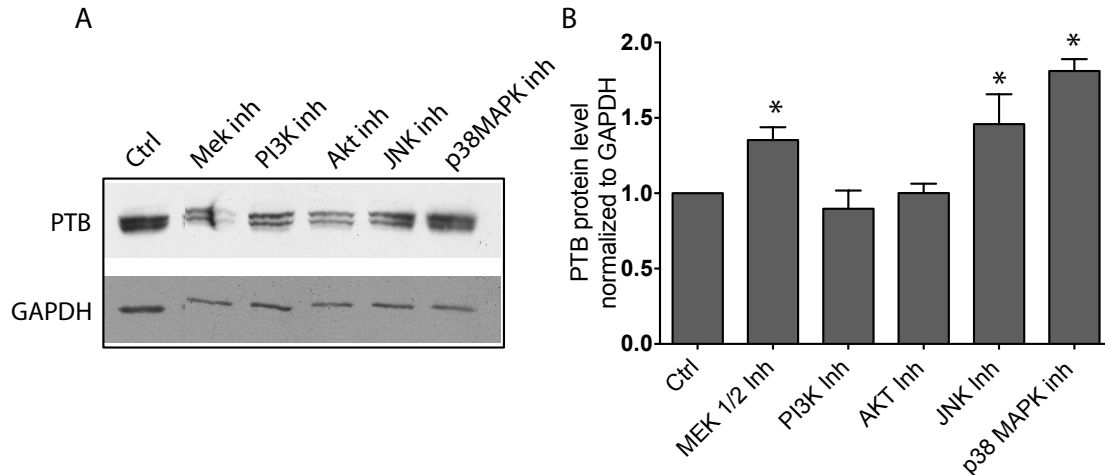
Figure 4.13. **Expression of PTB protein is differentially affected by pharmacologic inhibition of various kinases in the PI3K and MAPK signaling pathways.** Daoy cells were either untreated or treated with inhibitors to MEK1/2, PI3K, Akt, JNK, and p38 MAPK for three hours. (A) Western blotting was performed to detect PTB and GAPDH protein levels in untreated and inhibitor treated cells (C) The level of PTB protein was quantitated and normalized to GAPDH.

4.3C PTB protein level is reduced following mTOR inhibition by PP242

Finally I examined the impact of mTOR inhibition on PTB expression. Studies

have shown that PTB localization is regulated by amino acid availability, suggesting a role of

mTOR signaling in PTB regulation (Li & Yen, 2002). Analysis of PTB protein expression in

mTOR inhibited Daoy cells showed that PTB expression was reduced by 40% in PP242

treated cells compared to untreated cells (Figure 4.14 A, B). This result suggests that the

increased tau IRES activity in PP242 treated cells could be due to derepression of tau caused

by reduction of PTB. I have previously shown that reduced PTB protein level also allows the

proper splicing and translation of nPTB. However, PTB reduction by PP242 did not induce

the expression of nPTB (Figure 4.14A). Since I showed that PP242 also increased APP

IRES mediated translation, I examined whether the APP ITAF La protein was affected by

mTOR inhibition as well. Indeed, PP242 treatment increased La protein expression by 1.2

fold (Figure 4.14A, B).
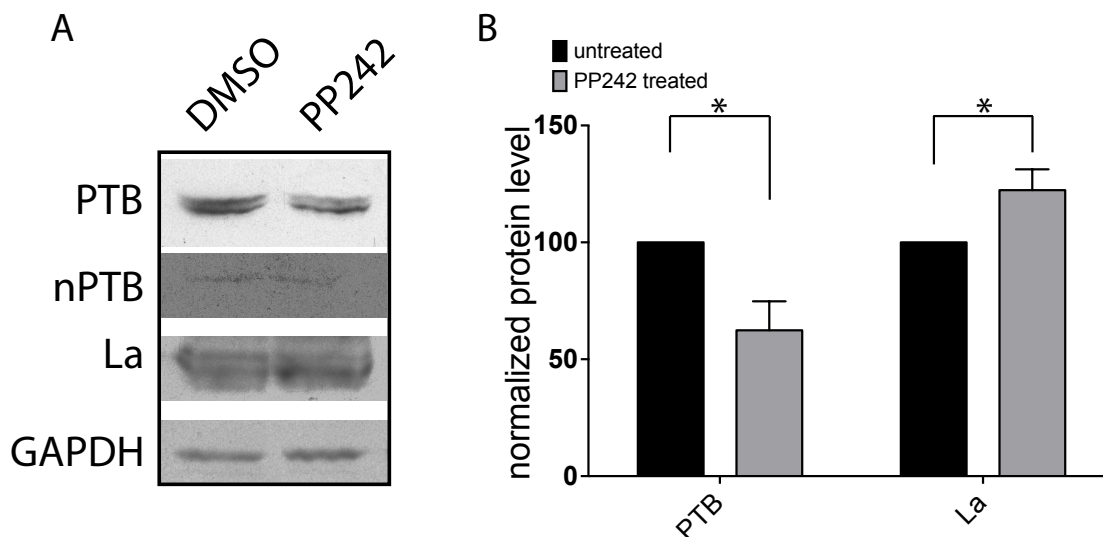
Figure 4.14**. PTB protein level is reduced following mTOR inhibition by PP242.** Daoy cells were either untreated, or treated with 250 uM PP242 for three hours. (A) Western blotting was performed to detect the level of PTB, nPTB, La, and GAPDH proteins. (B) The level of PTB and La proteins in untreated and PP242 cells were quantitated and normalized to GAPDH levels. Error bars represent SD. * $p < 0.004$.

These results suggest that the altered ITAF expression is one of the causes leading to enhanced tau and APP IRES activity after mTOR inhibition. As mentioned before, tau IRES activity is not solely dependent upon PTB expression. Further examination of other factors involved in regulating tau IRES activity is required.

4.4 Regulation of tau IRES-dependent translation by 3' UTR sequences

Along with sequences in the 5'UTR*, cis* elements in the 3' UTR can mediate protein synthesis by various mechanisms including altering mRNA stability, localization, ribosome accessibility, mRNA circularization and access to translational machinery such as regulation by miRNAs (Chatterjee & Pal, 2009). An iron responsive element (IRE) is present in the 5'UTR of amyloid precursor protein (APP) that allows for the regulation of APP expression in response to extracellular iron levels (J. T. Rogers et al., 2002). Similarly, APP 5'UTR also contains interleukin-1 (IL-1) responsive elements that regulate APP translation

in response to IL-1α and IL-1β (J. T. Rogers et al., 1999). Moreover, 5'UTR and 3'UTR can have micro-RNA (miRNA) binding sites that can modulate protein expression (Smith et al., 2011). The role of mRNA 3'UTR in IRES dependent translation is understudied and as result unclear.

During cap-dependent translation, sequences in the 3'UTR either directly bind to sequences within the 5'UTR or interact indirectly through RNA binding proteins such as PABP, which can facilitate the 5'-3' UTR interaction to enhance translational efficiency. Recent studies have indicated that the 3' UTR also influence 5' UTR mediated IRES activity. Both viral and cellular IRESs such as HCV and HIF1-α have been shown to have enhanced IRES activity in the presence of their respective 3' UTRs (Woeller et al., 2007) (Galban et al., 2008). For example the HIF1-α IRES utilizes PTB to bind the 3' UTR and facilitate circularization of the mRNA by binding to an additional ITAF HuR, which is associated with the 5' UTR (Galban et al., 2008).

The 3'UTR of tau is very large (4163 bp) and has been previously demonstrated to influence translation of tau mRNA, although the mechanism is unclear (Atlas, Behar, Sapoznik, & Ginzburg, 2007; Behar, Marx, Sadot, Barg, & Ginzburg, 1995). The experiments shown in this section demonstrate that the tau 3' UTR promotes translation mediated by the tau leader.

4.4A Tau 3'UTR enhances tau IRES activity

In order to assess the regulation of tau IRES dependent translation by the tau 3'UTR, I generated a series of *Photinus* luciferase constructs containing the tau, AurA, or β-globin 5' UTR sequences with the respective 3' UTR sequences downstream of the *Photinus* ORF (see methods and Figure 2.1). Addition of the tau 3' UTR sequence resulted in a significant increase (~6 fold) in tau IRES-mediated translation (Figure 4.15 A). Conversely, the addition of AurA, or β-globin 3' UTR did not affect IRES-dependent translation by their respective 5' leaders.

To further examine the specificity of the interaction between the tau 5' and 3' UTRs, the tau 3' UTR sequence was placed downstream of the *Photinus* ORF containing AurA or β-globin leaders. In these contexts the tau 3'UTR did not enhance IRES-mediated translation mediated by the AurA or β-globin leaders (Figure 4.15 B). These results demonstrate that there is specific interaction between the 5' and 3' UTR of tau mRNA, which enhances internal translation by the tau 5' UTR.
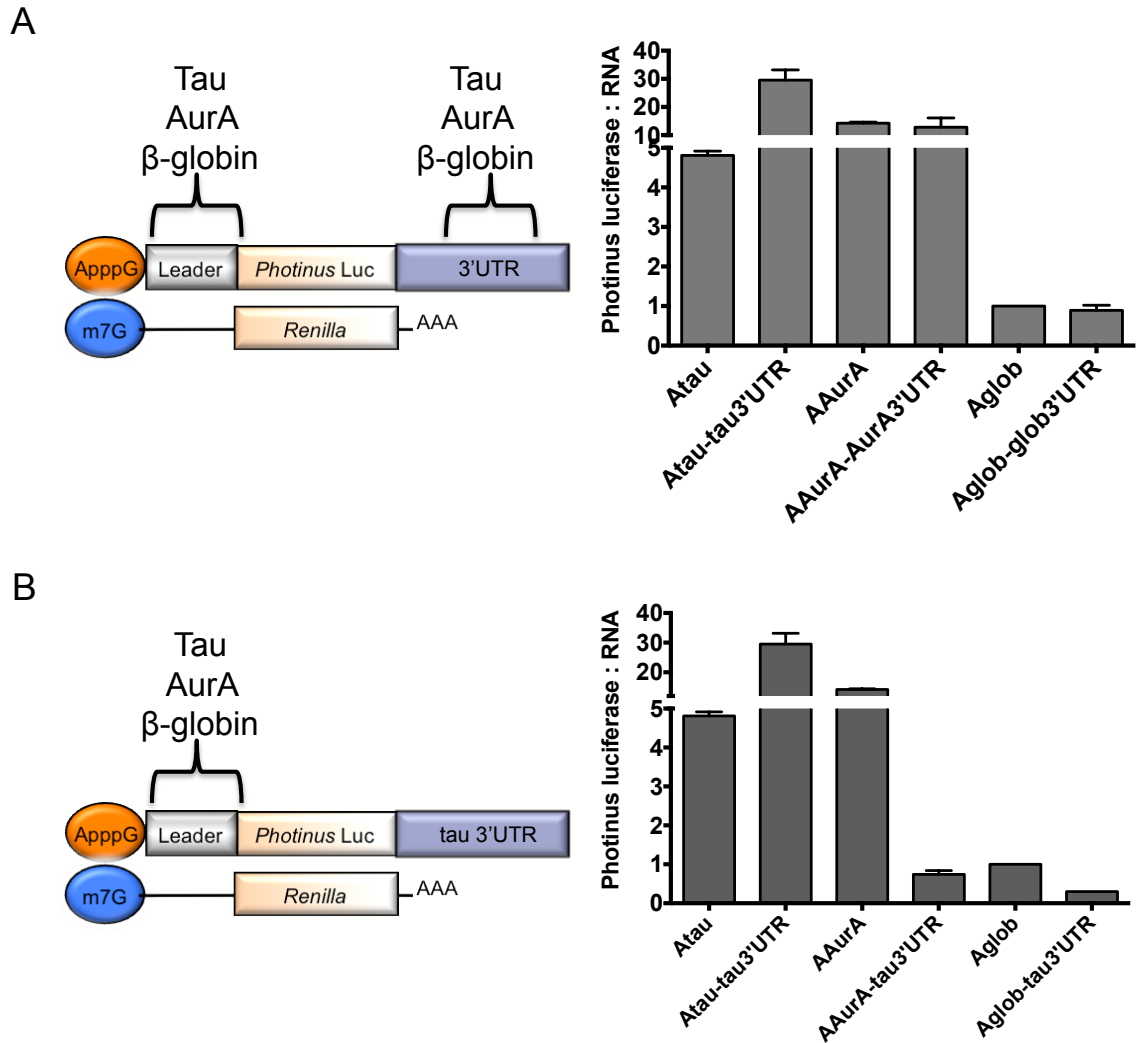
Figure 4.15. **Tau 3'UTR enhances tau IRES activity. (**A) 3' UTR sequences of tau, β-globin, or AurA were cloned downstream of the *Photinus* luciferase ORF with the corresponding 5' leaders upstream of the ORF. MRNAs were *in vitro* transcribed, capped with an ApppG capped, poly (A) tailed and cotransfected into Daoy cells along with m7G capped *Renilla* luciferase mRNA. Cells were then used in a dual luciferase assay, or harvested for total RNA, and RTPCR was performed using *Photinus* primers. A ratio of P:RNA was determined and normalized to the value obtained from mRNA with β-globin 5' leader only. (B) 3' UTR sequences of tau was cloned downstream of the *Photinus* luciferase ORF with the eithe rtau, β-globin, or AurA 5' leaders upstream of the ORF. MRNAs were transfected as in the above experiment, and P:RNA was normalized to the value obtained from mRNA with the β-globin 5' leader. (n=3). Error bars represent SD.

4.4B Full length tau 3'UTR is required for interaction with the tau IRES

I sought to identify the regions in tau 3' UTR that are essential for interaction with the 5' UTR. I generated a series of constructs containing either 3' or 5' deletion of the 3' UTR (shown in Figure 4.16 A). Deletion of 3750-4000 bp sequences in the 5' end of the 3' UTR, or deletion of 1000-4000 and deleting the 3' end of the 3'UTR –even up to 3Kb-maintained tau IRES activity to at control (only 5'UTR) levels. Interestingly, addition of only the 500bp or 163 bp of the 3'end of the 3'UTR greatly diminished IRES activity compared to the 5'UTR only control (Figure 4.16 B). These results imply that the full length 3' UTR is required for interaction with the 5'UTR, possibly through the sequences in its 5'end.
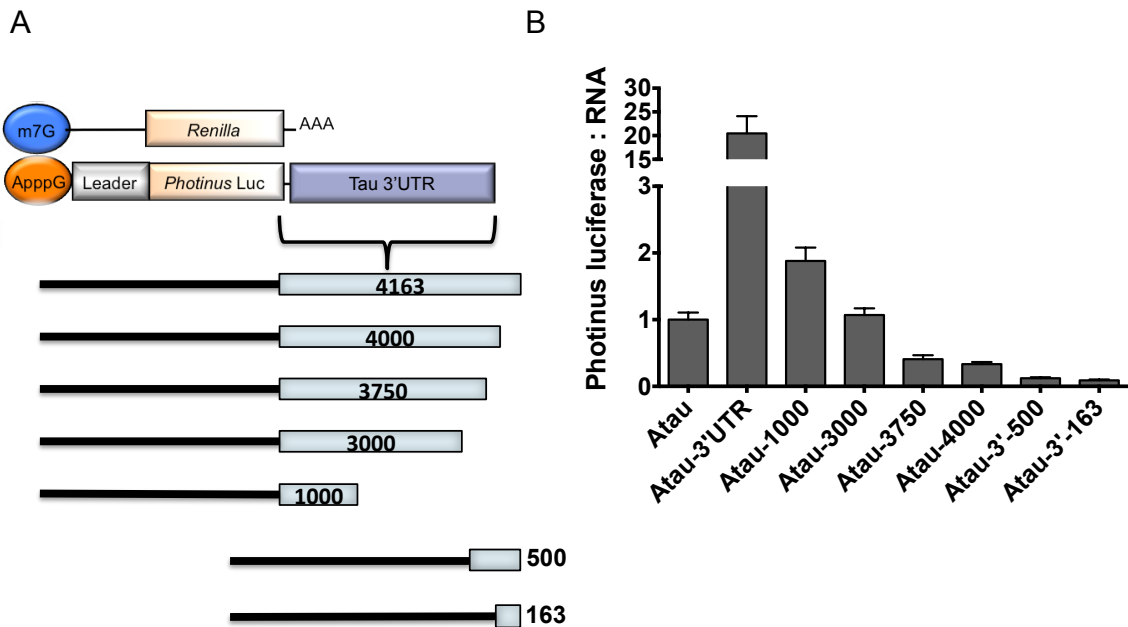
Figure 4.16**. Full length tau 3'UTR is required to enhances tau IRES activity.** (A) Schematic of the mRNAs used showing the full length tau 3' UTR (4163bp) and the various deletion mutations in the 3' end (1000, 3000, 3750, 4000) and the 5' end (500, 163). (B) ApppG capped *Photinus* luciferase mRNA with tau 5' leader and the various 3' UTR sequences (shown in A) was cotransfected along with m7G capped *Renilla* luciferase mRNA. Cells were then used in a dual luciferase assay, or harvested for total RNA, and RTPCR was performed using *Photinus* primers. A ratio of P:RNA was determined and normalized to the value obtained from mRNA with tau 5' leader only. (n=3). Error bars represent SD.

The deletion analysis suggests the presence of multiple regulatory elements. Deletion of the last 163 nucleotides of the 3'UTR results in both lost of enhanced IRES translation, but also inhibition of steady-state levels compared to the full length 3'UTR. This suggests that the deletion may activate a repressor sequence. IRES-mediated translation is restored to control levels with the deletion of an additional 1000 nucleotides placing the inhibitor between 3000-4000 nucleotides. Some enhancer activity was restored by further deletion as observed in the Atau1000 construct, suggesting the unmasking of an additional inhibitory element. Because deletion experiments suggested the localization of the enhancer to the last 163 nucleotides I sought to see if this region was sufficient for activation of IRES-mediated translation. The data show that this 163 nucleotide region alone, as well as, a larger 500 nucleotide region was incapable of restoring enhancer activity. These results are most consistent with a complex enhancer requiring sequences from both ends of the 3'UTR or the requirement of a complex secondary structure that cannot be recapitulated by the short sequence allow. Unfortunately, I cannot distinguish between these possibilities or changes that may result from differences in mRNA stability.

4.4C PTB knockdown further increases tau 3'UTR induced enhancement of tau IRES activity

For some IRESes, PTB promotes IRES activity by either directly binding to the 3' UTR, or by interacting with other proteins bound to the 3' UTR. I examined whether PTB is involved in facilitating the tau 5'-3' UTR interaction by analyzing tau 5'-3'UTR mediated IRES activity in PTB knockdown cells. PTB reduction further enhanced the previously increased translation by the tau 5'-3' UTR interaction (Figure 4.17). These results show that

PTB not only binds the tau 5' leader to inhibit IRES activity, but it also plays a role in tau 5'-3' interaction.
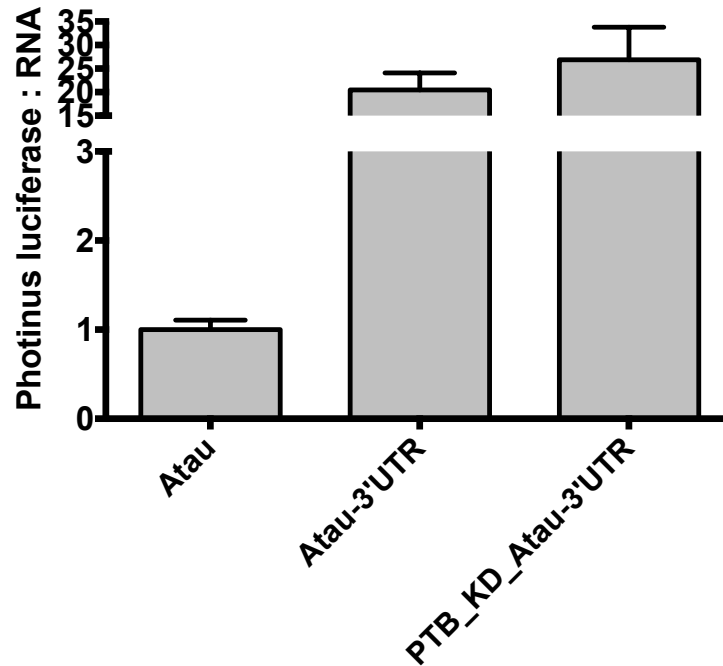
Figure 4.17. **Knockdown of PTB further enhances tau 3' UTR mediated increase of IRES activity.** ApppG capped *Photinus* lucifease mRNA with tau 5' leader with or without the the full length 3' UTR sequences was cotransfected into control or PTB knockdown cells along with m7G capped *Renilla* luciferase mRNA. Cells were then used in a dual luciferase assay, or harvested for total RNA, and RTPCR was performed using *Photinus* primers. A ratio of P:RNA was determined and normalized to the value obtained from mRNA with tau 5' leader only. (n=3). Error bars represent SD.

Summary

In this chapter I identified two proteins- PTB and nPTB , that function as inhibitory ITAFs for the tau IRES. I showed that PTB and nPTB bind the tau 5' leader sequence, and not only inhibited tau IRES dependent translation, but also negatively regulated endogenous tau protein expression. There are multiple regions within the tau IRES that PTB and nPTB could potentially bind. Because of the limited analysis I was unable to identify the region in the tau 5' leader that is important for PTB binding. Instead, as any mutation made to the tau 5' leader disrupted the secondary IRES structure and abolished IRES activity, my results suggest that along with the 5' UTR sequence, the secondary RNA structure as well as tertiary RNA interactions may be important in regulating tau IRES activity. Indeed, our previous studies have shown that small deletions and mutations disrupting tau IRES structure reduced the IRES activity, while compensatory mutations that rescued the structure then recovered the decreased IRES dependent translation. It is likely that a correct confirmation of the RNA is required before PTB, or nPTB could bind the tau 5' leader. This could be achieved either by the interaction among the sequences in the 5' leader itself, or with the help of additional factors such as other ITAFs, or sequences in the 3' UTR. One of these factors could be the PCBP2 protein. My preliminary data suggest that reduction of PCBP2 does not affect tau protein expression or IRES activity. However, I could only achieve a partial knockdown of PCBP2 in these cells. It is possible that further reduction of the protein is required before any changes in tau expression or IRES activity can be observed. Additionally, analysis of binding of PCBP2 to tau IRES is required before eliminating PCBP2 as a candidate factor.

For many IRESes, the interaction between the 5', and 3' UTR is facilitated by binding of a single accessory protein to both the 5' and 3' UTRs, or by interaction between

multiple ITAFs that bind either the 5', or the 3' UTR sequences. Supporting this theory, I showed that the sequences in the tau 3' UTR enhanced IRES-mediated translation mediated by the 5' leader. My results demonstrated that the entire 3' UTR was required for interaction with the tau 5' leader. In addition, I showed that removal of PTB further enhanced the 3' UTR mediated increase of tau IRES activity. It is possible that PTB binds to sequences in the 3' UTR as well and acts as a bridge to connect tau 5' and 3' UTRs. Alternatively, there could be other factors that bind to the 3' UTR, which can interact with PTB bound to the 5' leader and form a complex to inhibit tau IRES dependent translation. RIP experiments to identify factors associating with the 3' UTR could be performed to help identify these novel proteins.

Another factor that regulates IRES dependent translation is the differential expression of ITAFs in different cellular conditions. Examination of the various conditions in which tau IRES dependent translation is upregulated or downregulated showed that changes in PTB expression did not always correlate with the changes in tau IRES activity. During mTOR inhibition by PP242, PTB expression was reduced with a concomitant increase in tau IRES activity. However, during FAC or poly (I:C) treatment, tau IRES dependent translation was increased without any observable changes in PTB protein expression. It is possible that these conditions led to nuclear translocation of PTB or reduced PTB binding to the IRES without affecting PTB expression. Another possibility is that perhaps other tau ITAFs are expressed during these conditions, which then promote tau IRES dependent translation.

Unfortunately, I was unable to identify a positive ITAF for tau IRES in this study. However, I demonstrated for the first time that both PTB, and nPTB function as negative regulators of the tau IRES, and uncovered a role for the 3' UTR in tau IRES mediated translation.

Chapter V

Discussion and

Future Directions

5.1 Why study the regulation of tau IRES

Cap-dependent translation initiation is the main mechanism for translation of an mRNA. Most cellular mRNA possess the 5' m^{7}G cap structure, which can be recognized by the cap binding protein eIF4E to initiate protein synthesis. There are subsets of mRNAs that are inefficient at initiating translation by the cap-dependent mechanism. Many of these mRNAs contain long 5' leaders that are often G/C rich, suggesting presence of strong secondary RNA structure. IRES mediated translation is an alternate mechanism utilized by a subset of mRNAs in addition to cap-dependent translation. IRES-containing mRNAs usually code for proteins involved in important cellular processes such as proliferation, apoptosis, mitosis, etc, and often allow proteins to be translated under conditions when cap-dependent translation is inhibited. In addition, these specialized mRNAs utilize IRES-mediated translation for a precise and specific control of protein synthesis to regulate their expression temporally and spatially during various cellular conditions where other mechanisms may not be available. Previous studies have shown that the MAPT 5' UTR contains an IRES (Veo & Krushel, 2009). In this study, I investigated how the IRES dependent translation of tau is regulated.

The tau protein is abundantly expressed in the brain and normally functions to control microtubule dynamics. Our previous studies have shown that the tau mRNA 5'UTR contains an IRES that is capable of initiating cap-independent translation in SKNSH human neuroblastoma cells. In this thesis, examination of tau IRES activity showed that it is utilized by other neural cell types (U87 human glioma, Daoy human medulloblastoma, and C6 rat glial cells) as well as non-neural cells (MCF12A human breast epithelial). In fact, a monocistronic reporter assay indicated that the IRES is used for up to 50% of translation tau

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mRNA in U87 cells. My results suggest that IRES dependent translation is an essential mechanism utilized by the tau mRNA to regulate its protein synthesis in neural and non-neural cells.

5.2 Signaling pathways regulating IRES dependent translation

The identification of stimuli and pathways can provide information regarding the cellular conditions in which a given IRES is utilized. Since the internal translation of many IRES containing mRNAs is upregulated during cellular stress conditions such as oxidative stress, hypoxia etc, in this study I focused on examining the regulation of tau IRES activity during stress conditions commonly observed in NDs. An upregulation of tau IRES activity was observed during iron induced oxidative stress, and dsRNA infection by poly (I:C). However, cellular conditions mimicking ischemia did not affect tau IRES activity, but led to an increase in APP IRES activity. These results suggest that the tau IRES is regulated by some stress conditions observed in NDs. However, in NDs, multiple stress conditions are concurrently present. Perhaps, examination of tau IRES activity during a combination of stress such as ischemia and oxidative stress could provide a better indication about the use of tau IRES in disease pathology.

The regulation of tau is well studied in Alzheimer's disease. One of the characteristic features of AD is the presence of extracellular Aβ plaques that are composed of cleaved APP protein. APP mRNA contains an IRES as well. A comparison of tau and APP IRES activities revealed that addition of exogenous Aβ peptides increased both tau and APP IRES dependent translation. Increased APP IRES activity was also observed during iron induced oxidative stress, poly (I:C) treatment, and chemical ischemia. I previously showed that increased APP IRES activity leads to elevated APP protein level after the addition of iron and poly (I:C)

(Beaudoin et al., 2008). Tau protein levels remained unaffected during these conditions. My results highlight the importance of tau protein regulation and suggest that tau IRES plays an important role to maintain the level of the protein during normal, and pathogenic conditions. Indeed, regulation of protein synthesis is a much more efficient mechanism to regulate the expression of protein compared to transcriptional regulation. Having an IRES allows some specific mRNAs to have an additional control over their protein expression. The tau IRES likely functions as an important regulator of tau protein expression in response to stress conditions in the brain.

Signaling pathways regulating specific cellular IRESes have been previously demonstrated. For example, the AKT pathway negatively regulates cyclin D1 and the c-myc IRES, while rapamycin treatment enhances IRES use in a p38 MAPK and RAF/MEK/ERK signaling-dependent manner (Shi, Sharma et al. 2005). In this study I examined signaling molecules and pathways that are involved in regulating tau IRES-dependent translation. Since both cap-dependent and IRES-dependent pathways utilize many of the same molecules and factors for translation, components of the MAPK, and PI3K-mTOR pathways were tested. These pathways are activated by various upstream stimuli such as growth factors, cytokines etc. Addition of several growth factors (EGF, IGF-1, BDNF) reduced translation by the tau IRES, while other factors (insulin, FGF2) did not affect tau IRES activity. Further, the reduced activity by EGF, IGF-1, and BDNF was independent of the increase in cap-dependent translation by these factors. Unlike the global regulation observed for cap-dependent translation, these growth factors differentially regulated specific IRESes. For example, addition of IGF-1 reduced tau IRES activity, but it increased APP IRES activity. In addition, neurotrophic factors also differentially regulated tau and APP IRES-dependent

translation. GDNF, Hrg-1β, and neurturin reduced tau IRES activity but increased APP IRES activity. My results indicate that these growth factors likely function via MAPK and PI3K pathways. However, my results show that the regulation of different IRESes by different growth factor is specific and cannot be generalized. This suggests that the tau IRES is influenced by various upstream stimuli, but the specific regulation of tau expression by its IRES likely occurs downstream of these pathways.

Pharmacological inhibition of various kinases in the MAPK and PI3K pathways reduced both cap-dependent and IRES dependent translation of both tau and APP IRESes. These results demonstrated that different cellular cues modulate both modes of translation, through similar pathways, likely through the mTOR signaling. The mTOR kinase controls cap-dependent translation directly through the mTORC1 complex, and indirectly through the mTORC2 complex. Tau and APP protein expression was decreased following siRNA-mediated knockdown of mTORC1 and mTORC2 proteins Raptor and Rictor respectively. Tau (but not APP) IRES activity was also reduced with Raptor knockdown, while knockdown of Rictor had no effect on IRES mediated translation of tau or APP. Raptor is an essential protein in the mTORC1 complex. Sustained knockdown of mTOR might have unknown effects that may directly affect tau IRES dependent translation, or affect other factors of the translational machinery. Alternatively, knockdown of Raptor (and Rictor) might even induce apoptotic signaling, which may then have a role on tau translation and protein expression. Acute inhibition by pharmacological drugs can provide a better answer regarding the role of mTOR pathway in tau IRES dependent translation. In my studies, inhibition of mTOR signaling by its active site inhibitor PP242 showed that despite diminishing cap-dependent translation, tau (and APP) proteins were continually translated,

and that during mTOR inhibition, IRES-dependent translation is utilized to maintain tau (and APP) protein expression.

mTor is a global regulator of cap-dependent translation. Therefore, it is possible that inhibiting cap-dependent translation by mTOR inhibitors may facilitate translation of IRES containing mRNAs. Indeed, previous studies have shown that cyclin D1 and c-myc IRES activity is enhanced following exposure to mTORC1 inhibitor rapamycin(Shi et al., 2005). One explanation could be that inhibiting cap-dependent translation frees up ribosome and the translational machinery to be utilized for IRES-dependent translation. However, translation of specific IRES still requires additional regulatory mechanisms.

A screen of human kinases revealed four proteins ULK2, PRKX, CHK2, and EIF2AK2 as potential regulators of tau IRES dependent translation. Unfortunately, I was unable to repeat the results for two of the four strongest hits (ULK2, and PRKX) from the screen in separate experiments. Different sets of siRNA for the proteins were used between the experiments, which could have resulted in the disparity in the results. Further analysis of the data from the siRNA screen showed that siRNAs to other kinases (such as kinases in the MAPK, and PI3K pathways, that were shown to regulate tau IRES activity) did not affect tau IRES activity. It is possible that the siRNAs in the screen did not completely knockdown the kinases. In addition, it might be necessary to knockdown a complete pathway and not just a single kinase. Some of the inhibitors used in my assay target multiple kinases, and therefore the combined effect might be more pronounced than from a single kinase knockdown. Further, this screen was performed in SKNSH cells, in the background of normal cap-dependent translation. In my examination of IRES usage in different cell lines, tau IRES was utilized only up to 30% in SKNSH cells during normal cellular conditions. Kinases that

decrease the IRES activity could decrease by only up to 30%, while an increase in IRES activity by these kinases would be restricted. This means that small increases /decreases in IRES activity would be hard to detect using these cells. Performing the screen in other cell lines with better tau IRES usage (such as Daoy, or U87 cells) in normal cellular condition as well as in conditions that increase the tau IRES activity might provide better information regarding regulators of tau IRES.

5.3 Tau ITAFs: are there others ?

While identification of signaling pathways and network regulating an IRES can provide some information about the physiological function of the IRES, an overarching question in the field is how these upstream signals cue the IRES translational machinery to modulate its activity. One mechanism by which upstream signals can be transmitted is through non-canonical proteins called IRES-transacting factors (ITAFs). ITAFs are RNA binding proteins that bind specific sequences (or specific RNA structure) within the IRES. Expression level of ITAFs is thought to be the rate-limiting step for eukaryotic IRESes. In this study, I was able to identify two proteins- PTB and nPTB, which function as inhibitory ITAFs for the tau IRES. PTB and nPTB bound the tau 5' leader sequence, and not only inhibited tau IRES dependent translation, but also negatively regulated endogenous tau protein expression. The binding of PTB and nPTB to the tau IRES was specific, as PTB, or nPTB did not bind to the APP IRES sequence. La protein, however, bound APP, but not tau IRES.

There are multiple regions within the tau IRES that PTB and nPTB could potentially bind. Examination of mutations made to the tau 5' leader abolished IRES activity. However, I cannot conclude that disruption of PTB binding inhibits tau IRES activity. The

Tau IRES is highly structured and maintaining the structure is crucial for its IRES activity. Previous work from our lab has shown that small mutations and deletions in the tau leader that affect the IRES structure reduce the IRES activity, while compensatory mutations that rescued the predicted structure functioned to rescue the decreased IRES dependent translation (Veo & Krushel, 2012). It is possible that these mutations intended to reduce PTB binding also altered the secondary structure of the IRES such that the IRES can no longer initiate translation. It would be important to examine whether PTB still binds to these sequences. In addition, experiments that examine the secondary structure of the leader with the mutations are required to confirm these results. RNA structure rather than primary sequence may be required before PTB, or nPTB binds to the tau 5' leader.

Current models propose that binding of an enhancer ITAF to an IRES facilitates translation by either directly recruiting the translational machinery or by changing the conformation of the RNA to promote ribosome binding. The precise mechanism by which inhibitory ITAFs regulate translation is not known. To date, only a handful of inhibitory ITAFs have been identified. There are several possible mechanisms through which these ITAFs could inhibit IRES dependent translation. First, these inhibitory ITAFs could compete with positive ITAFs for the same binding sites within an IRES, yet lack the ability to activate translation, for example through recruitment of the ribosome of the translational machinery. Such a model is supported by the observation that some IRESes have been shown to bind multiple ITAFs, both positive and negative, and there are specific examples of competition for binding to the same sequence with the IRES. Such a model is consistent with a regulatory pathway that allows for both activation and repression of IRES use. For example, PTB, hnRNPA1, and PDCD4 proteins function as inhibitory ITAFs for the XIAP IRES,

while La, hnRNPC1, and MDM2 stimulate its IRES activity (Holcik, Gordon, & Korneluk, 2003; Holcik & Korneluk, 2000; Lewis et al., 2007; T. Liu et al., 2015; Liwak et al., 2012; Roy et al., 2014). In such a case, the subcellular localization and expression level of the ITAFs control the IRES activity. In addition, PTM of ITAFs could play a role by influencing their cellular localization and RNA binding activity. Other factors such as cellular conditions also influence ITAF sub cellular localization and expression. For instance, PTB and Unr proteins negatively regulate the UNR IRES. However, during mitosis, hnRNPC1/C2 proteins migrate to the nucleus and bind the IRES to promote IRES dependent translation of Unr. It is likely that binding by hnRNPC1/C2 causes a conformational change in the UNR IRES structure which impedes binding by PTB and Unr proteins, thus relieving the inhibitory effects by PTB and Unr (Schepens et al., 2007). It is possible that a similar mechanism exists for tau IRES and stimulatory tau ITAFs only bind during specific cellular conditions where enhanced tau expression is required.

Using the candidate factor approach, I was unable to identify an ITAF that functioned to enhance use of the tau IRES, however, cannot rule out the existence of one. First, the candidate proteins were chosen based on the presence of their known binding sites within the tau IRES. Thus I excluded proteins that could bind the IRES through a specific RNA structural motif. In addition, the functional analyses of the candidate ITAFs were performed during normal cellular conditions. As previously mentioned, the expression, and subcellular localization of a given ITAF is modulated by the activity of various signaling molecules in response to changes in cellular conditions. Therefore, it remains possible that specific ITAFs were not tested under conditions where they were functionally active. An

unbiased approach to identify proteins that bind the tau IRES could better help identify other novel tau ITAFs.

Examination of the various conditions in which tau IRES-dependent translation is upregulated or downregulated showed that changes in PTB protein levels determined by western blot analysis did not always correlate with the changes in tau IRES activity. During mTOR inhibition by PP242, PTB expression was reduced with a concomitant increase in tau IRES activity. However, during FAC or poly (I:C) treatment, tau IRES-dependent translation was increased without any observable changes in PTB protein expression. However, it is important to note that measurement of steady-state PTB levels may not accurately reflect its role in IRES regulation. It is possible that these conditions affect PTB binding to the IRES without affecting PTB protein levels. PTB-dependent enhancement of viral IRESes occurs without changes in steady-state protein. Perhaps, it is more important to assess whether PTB binding to the tau IRES changes during these various conditions. In addition, changes in subcellular localization or perhaps recruitment by other ITAFs may be critical for PTB action on tau IRES. It is also possible that the presence of tau enhancer ITAFs expressed during these conditions overcome the inhibitory effects of PTB to promote tau IRES dependent translation. One of these co-factors could be another candidate ITAF PCBP2. siRNA mediated knockdown of PCBP2 led to only a partial reduction of the protein. It is possible that the remaining PCBP2 protein is sufficient for binding to the IRES or for interacting with PTB or other putative tau ITAFs. The level of PCBP2 protein in different stress conditions was not examined. Perhaps PCBP2 binds to the tau IRES only in specific cellular environment. A detailed analysis of PCBP2 expression under conditions in which tau IRES is upregulated/downregulated is required to sort these various possibilities out.

For some IRESes, the same ITAF can function to promote as well as inhibit IRES activity. For instance, hnRNPA1 enhances IRES dependent translation of cyclin D1 (Shi et al., 2005), however, Akt-dependent phosphorylation of hnRNP A1 inactivates it ability to stimulate its IRES activity (Jo et al., 2008). A similar mechanism might exist with PTB for tau regulation. Indeed, the phosphorylation status of PTB is important for its subcellular localization. PTB is phosphorylated by PKA at Ser-16, which promotes its nuclear export and accumulation in the cytoplasm. PTB with mutated Ser-16 site accumulates in the nucleus (J. Xie, Lee, Kress, Mowry, & Black, 2003). Perhaps, during conditions when tau IRES is upregulated, PTB is translocated to the nucleus, thereby allowing the tau IRES to initiate translation. It is also possible that these cellular conditions modify PTB which reduces its binding to the tau IRES without affecting PTB expression.

In addition to the sequences in the 5' UTR, sequences in the 3' UTR of the mRNA have been shown to play a role in protein translation. The PABP, which is bound to the 3'UTR of mRNAs, interacts with eIF4E (bound on the 5' end) through its interaction with eIF4G. This circularizes the mRNA thereby increasing mRNA stability but also enhancing stability of translational machinery binding, thus enhancing translation (Jackson et al., 2010; Sonenberg & Hinnebusch, 2009). My study showed that the sequences in the tau 3' UTR enhanced IRES dependent translation mediated by the 5' leader. Further, the entire 3' UTR was required for interaction with the tau 5' leader.

For some IRESes, binding of a single accessory protein to both the 5' and 3' UTRs facilitates the interaction between the 5', and 3' UTR. For example, PTB binds both the 5' and 3' UTRs of coxsackievirus B3 virus and enhances its IRES dependent translation likely by promoting RNA circularization (Verma, Bhattacharyya, & Das, 2010). Alternatively,

different ITAFs could bind either the 5', or the 3' UTR sequences and facilitate IRES activity

by protein-protein interaction. For instance, the HIF1-α IRES is enhanced by interaction

between PTB, which binds to its 3' UTR, and another ITAF, which binds the 5' UTR

(Galban et al., 2008). In this study, I showed that PTB and nPTB bind the tau 5' leader

sequence. It is possible that PTB, and/or nPTB also binds sequences in the tau 3' UTR.

Further examination to identify proteins that bind to the 3'UTR is required before ruling out

PTB, or nPTB as the bridge between tau 5' and 3' UTRs. In fact, my results also showed that

removal of PTB further enhanced the 3' UTR mediated increase of tau IRES activity.

Perhaps there are other factors that bind to the 3' UTR, which can interact with PTB bound to

the 5' leader and form a complex to inhibit tau IRES dependent translation. RIP experiments

to identify factors associating with the 3' UTR can help identify these novel proteins.

5.4 Future Directions

One of the major challenges in studying translation mediated from an IRES

containing mRNA is demonstrating its physiological relevance. In this study, I showed tau

IRES utilization in neural and non-neural cells using monocistronic reporter transcripts.

Further, I demonstrated differential utilization of tau IRES in various cellular conditions.

However, much of the functional and mechanistic studies were done using normal cellular

context without the inhibition of regular cap-dependent translation. IRES dependent

translation of most (IRES containing) mRNAs is increased when cap-dependent translation is

shut down. I determined a few stress conditions in which tau IRES activity is upregulated.

However, how these conditions affect cap-dependent translation of tau is not clear. Since

these stress conditions are known to shut down cap-dependent translation, it is possible that

the increased IRES activity I observed during these conditions is a result of reduced cap-

dependent translation. A comparison of translation from the m7G versus A-capped tau RNA would provide a better explanation about the use of tau IRES during these conditions. Further, examination of how growth factors and signaling pathways affect tau IRES dependent translation during these conditions could uncover the pathways that specifically regulate tau IRES in a more physiological state. The same reasoning could be applied for the siRNA screen to identify additional kinases that regulates tau IRES activity under various cellular conditions.

Perhaps the most important question that remains to be answered is if any positive ITAFs are required to activate tau IRES translation, what those factors may be, and how they are regulated to control tau IRES ribosome recruitment. Unbiased methods to identify proteins that bind the tau IRES would help determine novel ITAFs for tau. It is likely that different ITAFs are utilized in different cellular conditions or in different cell types. Further, ITAF binding could be differentially regulated depending on the cell type. Certainly the identification of both PTB and nPTB as inhibitory ITAFs of tau validates this notion. The expression patterns of PTB and nPTB protein are generally mutually exclusive. This allows for the regulation of tau IRES in cells where either only PTB, or only nPTB is expressed. Other ITAFs could function in a similar manner and only bind to the IRES in specific cells. While these experiments demonstrated differential IRES use in many cell conditions and cell types, the search for ITAFs was limited to just a handful of known proteins with a putative binding site in the tau leader. Clearly, to increase the possibility of finding regulatory ITAFs it would be important to determine protein binding to the tau IRES in different cells, and in different cellular contexts by utilizing tagged RNA (such as a biotin tag) to crosslink and pull down the RNP complex followed by mass spectrometry to identify the proteins. A similar

approach could be used with tagged 3' UTR sequence to identify proteins binding to the 3' end. These assays would need to be followed by functional analysis to establish their role as an ITAF.

It remains possible that PTB (and nPTB) might be the only ITAF regulating tau IRES utilization. One model could be that the IRES secondary structure is sufficient to recruit translation complex and that PTB-binding blocks this function. Thus activation would be the default pathway with inhibition actively regulated. Therefore, PTB would be a target of regulation. Examination of phosphorylation (as well as other post translational modifications) and localization of PTB in different cells and stress conditions could reveal if changes in PTB phosphorylation of localization correlate with changes in tau IRES activity.

5.5 Final words

IRES dependent translation is a unique tool utilized by a subset of mRNAs to regulate their protein expression. Having an IRES allows these mRNAs, including the tau mRNA, to control their protein levels in different conditions to meet the cellular requirement. There is currently a lot of effort being put into identifying mechanisms and factors that regulate all IRES dependent translation. While many IRESes might share some common mechanism or ITAFs for their regulation, I think more focus should be put on regulatory mechanisms for a specific IRES. Most cellular IRES containing mRNAs have important cellular functions. I believe that understanding the mechanism of how each of these IRESes are differentially regulated would not only provide us with a better understanding about the function of the protein but also provide us with unique opportunities to exploit this knowledge for therapeutic purposes.

Appendix
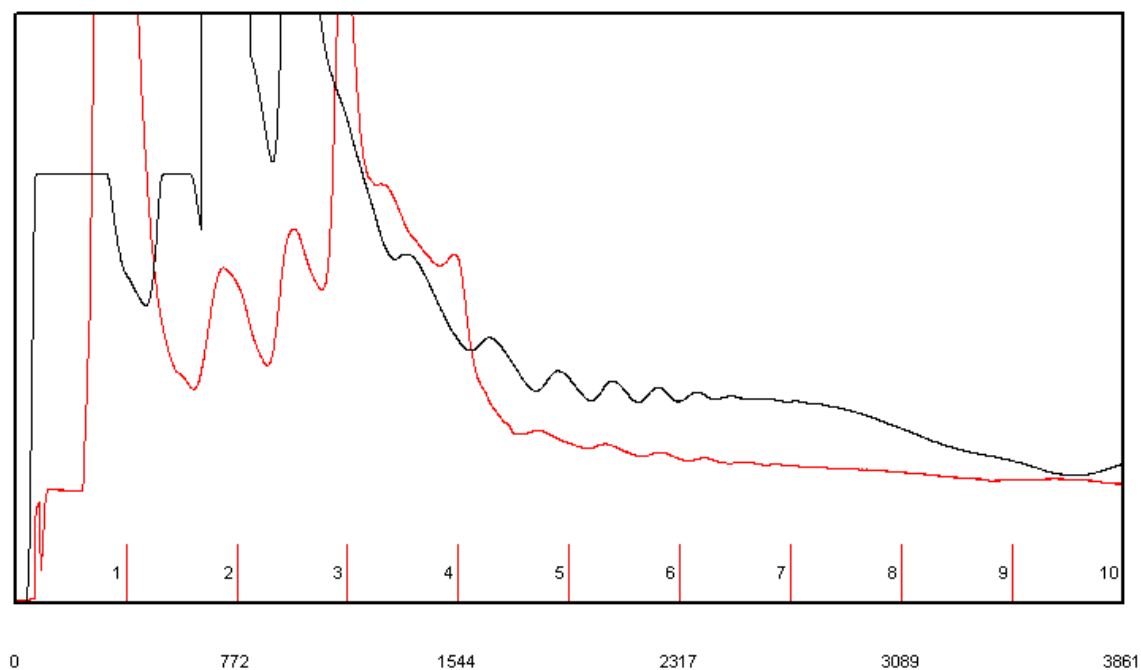
Appendix A1. **A representative graph of sucrose gradient fractions for DMSO treated (black) and PP242 treated (Red) samples.**

Appendix A2. **An average of P:R ratio for tau IRES following siRNA of human kinases (n=3).**

ITAF	Consensus binding site	Known IRES
La/SSB	GCAC	MYB, XIAP, LamB1
PCBP1/2	poly(C)	BAG1
PTB	C/U rich (CCU)n UCUU UCUUC	UNR, Xiap-1
nPTB	C/U rich	Apaf-1, MYB
hnRNP C1/C2	poly(C)	c-myc
hnRNP A1	UAGGGA/U	cyclinD1, c-myc, FGF2, VEGF, Apaf-1
hnRNP K	poly(C)	c-myc
Unr	AAGUA/G AACG	p58 PITSLRE
HuR	AU-rich	p27(Kip1), Xiap-1, IGF-IR

Appendix A3. **A list of candidate ITAFs for tau IRES with consensus binding sites and known targets.** IRESes that are stimulated are shown in purple, and IRESes that are inhibited are shown in red.

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